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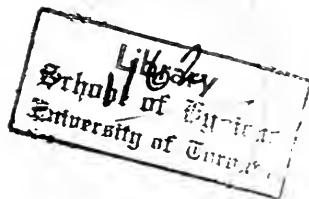
# *The* Journal of Infectious Diseases

Published by the John McCormick Institute for Infectious Diseases

EDITED BY  
LUDVIG HEKTOEN AND EDWIN O. JORDAN

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CONNAUGHT ANTITOXIN LABORATORIES  
RESEARCH DIVISION



Volume 29  
1921

Chicago, 1921

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J67  
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# THE IONIZATION CONSTANTS OF GLYCEROPHOSPHORIC ACID AND THEIR USE AS BUFFERS, ESPECIALLY IN CULTURE MEDIUMS

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The value of phosphates in culture mediums has long been recognized, and it has been specifically pointed out by Kligler<sup>1</sup> that their precipitation as it usually occurs, near the turning point of phenolphthalein, is most detrimental to the growth of certain organisms, notably streptococci and tubercle bacilli. With the idea of rectifying this difficulty, the employment of the glycerophosphates has already been suggested by one of us (Acree<sup>2</sup>) in a recent paper. One of us (Mellon) has used them with some apparent success as far back as 1912 in the cultivation of *B. acne*. The results, however, were never reported. So far as we know, their possibilities in culture mediums have not hitherto been recognized.

It is well known that calcium, magnesium and other glycerophosphates are soluble, in contrast to the insolubility of the phosphates. Table 1 shows the results of buffering nutrient beef broth, prepared in the usual way, with different concentrations of sodium glycerophosphate. There is slight or no precipitate on the acid side and but moderate on the alkaline side. With the potassium diacid phosphate there is a progressively increasing precipitate from  $P_H$  5 to 8, and it is even quite heavy at  $P_H$  7.

It is of interest that broth titrated with NaOH in the usual way precipitates in the cold on the alkaline side, but this precipitate may be dissolved by sodium glycerophosphate. When the broth, adjusted to  $P_H$  8, is autoclaved, a considerably heavier precipitate occurs than is true for that buffered with glycerophosphate, although it is not so marked as occurs in that buffered with  $KH_2PO_4$ . This must be true to an even greater extent with agar, which itself is known to contain salts of calcium and magnesium.

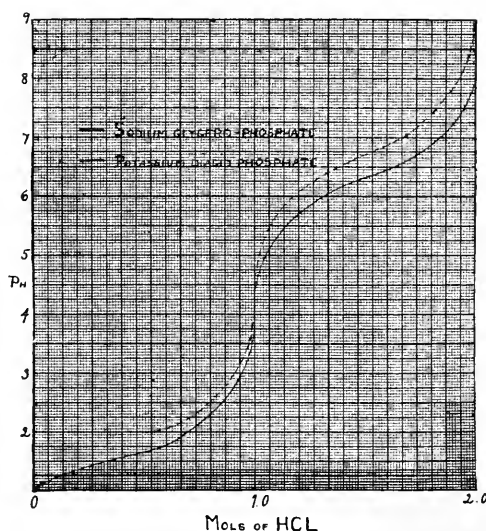
Received for publication Feb. 1, 1921.

<sup>1</sup> Jour. Bacteriol., 1917, 2, p. 351.

<sup>2</sup> Ibid., 1920, 5, p. 191.

It is observed with the  $\text{KH}_2\text{PO}_4$  series, that the  $P_H$  at 8 is changed after autoclaving. Such a change is also well known to occur in unbuffered mediums. This is in marked contrast to the mediums buffered at  $P_H$  8 with glycerophosphates in concentrations of M/10 and M/5 when no change in  $P_H$  occurs. A concentration of M/25, however, is not sufficient to prevent change in  $P_H$ .

In the past, attempts have been made to redissolve with HCl the precipitate formed in broth when neutralizing with NaOH. Although chemically the measure is partially successful, it has proved to be a



$P_H$  values for varying degrees of neutralization of the glycerophosphoric acid under the isohydric conditions employed.

deleterious practice from the standpoint of the nutritional requirements of certain organisms. When glycerophosphate is used as a buffer no precipitate forms, except under the conditions indicated in the foregoing.

The titration curve shown in the chart was obtained by titrating the disodium salt of alpha-glycerophosphoric acid with increasing quantities of hydrochloric acid up to two molecules. The readings were obtained with the hydrogen electrode. The curve gives the  $P_H$  values for varying degrees of neutralization of the glycerophosphoric acid under the isohydric conditions employed.



TABLE 1  
PRECIPITATION IN AUTOCLAVED BOUILLON BUFFERED WITH

pH	Na <sub>2</sub> C <sub>3</sub> H <sub>7</sub> O <sub>2</sub> PO <sub>4</sub>						KH <sub>2</sub> PO <sub>4</sub>						Control	
	1/5 Mol.		1/10 Mol.		1/25 Mol.		1/5 Mol.		1/10 Mol.		1/25 Mol.		No Buffer	
	PPT.	Fin. pH	PPT.	Fin. pH	PPT.	Fin. pH	PPT.	Fin. pH	PPT.	Fin. pH	PPT.	Fin. pH	PPT.	Fin. pH
5	0	5	0	5	0	5	1+	5	1+	5	1+	5	0	4.8
6	0	6	0	6	0	6	2+	6	2+	6	2+	6	0	5.4
7	0	7	1+	7	1+	7	3+	7	3+	7	3+	7	1+	6.6
8	2+	8	2+	8	2+	7.6	4+	7.4	4+	7.4	4+	7.4	3+	7.4

TABLE 2  
CALCULATION OF K<sub>1</sub> FOR GLYCEROPHOSPHORIC ACID

pH	H <sub>t</sub>	KAnH <sub>t</sub>	H <sub>2</sub> An	K <sub>1</sub> × 10 <sup>-2</sup>
2.3	5.01 × 10 <sup>-3</sup>	0.0306	0.00439	3.47
2.4	3.98 × 10 <sup>-3</sup>	0.0320	0.00402	3.02
2.5	3.16 × 10 <sup>-3</sup>	0.0333	0.00354	2.76
2.6	2.51 × 10 <sup>-3</sup>	0.0344	0.00309	2.53
2.7	2.00 × 10 <sup>-3</sup>	0.0354	0.00261	2.39
2.8	1.58 × 10 <sup>-3</sup>	0.0362	0.00222	2.26
2.9	1.26	0.0369	0.00184	2.18
3.0	10 × 10 <sup>-3</sup>	0.0374	0.0016	2.00
3.1	7.94 × 10 <sup>-4</sup>	0.0380	0.00121	2.13
Average.....				2.5

CALCULATION OF K<sub>2</sub> FOR GLYCEROPHOSPHORIC ACID

pH	H <sub>c</sub>	K <sub>2</sub> An <sub>t</sub>	KAnH	K <sub>2</sub> × 10 <sup>-7</sup>
5.0	10 × 10 <sup>-6</sup>	0.00246	0.03760	4.50
5.2	6.3 × 10 <sup>-6</sup>	0.0040	0.0360	4.98
5.4	4.0 × 10 <sup>-6</sup>	0.0060	0.0340	4.92
5.8	2.5 × 10 <sup>-6</sup>	0.0088	0.0312	4.96
5.8	1.6 × 10 <sup>-6</sup>	0.0120	0.0280	4.76
6.0	10 × 10 <sup>-7</sup>	0.0166	0.0234	4.96
6.2	6.3 × 10 <sup>-7</sup>	0.0216	0.0184	5.19
6.4	4.0 × 10 <sup>-7</sup>	0.0266	0.0134	5.53
6.6	2.5 × 10 <sup>-7</sup>	0.0300	0.0100	5.50
6.8	1.6 × 10 <sup>-7</sup>	0.0332	0.0068	5.42
7.0	10 × 10 <sup>-8</sup>	0.0355	0.0045	5.52
7.2	6.3 × 10 <sup>-8</sup>	0.0370	0.0030	5.45
7.3	4.0 × 10 <sup>-8</sup>	0.0376	0.0024	5.50
Average.....				5.2

The values of the ionization constants  $K_1$  and  $K_2$  were calculated by means of the approximate formulae developed in earlier papers on the use of the hydrogen electrode for measuring ionization constants, namely:

$$K_1 = \frac{H_t (L K_{AnH} + H_t)}{H_{tAn} - K_{AnH} - H_t} \text{ and}$$

$$K_2 = \frac{H_t (L' K_{sAn} + H_t)}{K_{AnH} - K_{sAn} - H_t}$$

$H_t$  is the concentration of total hydrogen ions,  $L$  is the degree of ionization of the acid salt and  $L'$  that of the neutral salt; and the other symbols give, as usual, the total concentrations of the ionized and nonionized acid, acid salt and neutral salt.  $L$  is taken as 0.83 and  $L'$  as 0.70, pending final values to be presented later. The columns in table 2 are self explanatory. The results were obtained colorimetrically.

The average values obtained for  $K_1$  as  $2.5 \times 10^{-2}$  and for  $K_2$  as  $5.2 \times 10^{-7}$  are close enough for  $K_1$  and  $K_2$  of phosphoric acid to warrant us in replacing the latter with the more desirable glycerophosphoric acid. For phosphoric acid,  $K_1 = 1.1 \times 10^{-2}$  and  $K_2 = 2 \times 10^{-7}$ , its relation to the glycerophosphoric curve being shown in the chart. Small corrections in  $P_H$  will enable us readily to compare past work on phosphoric acid buffers with the present work on glycerophosphoric.

On account of its constant weight and its relative stability, the anhydrous disodium glycerophosphate salt is the most utilizable for bacteriologic purposes. It can be completely dehydrated in vacuo or at 100 C. without decomposition. Sterilization in either the solution or solid form is preferably conducted at 100 to 110 C., owing to its tendency to decompose at higher temperatures. It has been shown by tests with  $CaCl_2$  that solutions of sodium glycerophosphate are slowly hydrolyzed under the above conditions into phosphoric acid salts. However, the dehydrated salt can be heated for days at 100 to 110 C. without giving enough phosphoric acid salt to yield more than the faintest opalescence by the  $CaCl_2$  test. Prolonged heating at 130 C. gives definite decomposition of 0.1 to 0.2 per cent. At 150 to 170 C. the salt gives off fumes and steadily loses weight, sodium phosphate resulting.

Cultures of various strains of streptococci and diphtheroids, as well as different members of the colon-typhoid-dysentery group, have grown at least as well, and in some cases better, than on the mediums ordinarily

employed by us. It would appear, then, that in concentrations of M/25 and M/10 no inhibition of growth has occurred. The work with cultures has been of a preliminary and general nature, with the exception of studies on the tubercle bacillus, which are about to be reported.

Experiments in this direction were prompted by the facts already related, together with the predilection of this organism for glycerin, as well as the fact that phosphates form at least 55 per cent of its ash. It is suggestive that a saprophytic strain of this organism appeared to show increased viability on this medium, particularly in the acid ranges. It does not replace the egg for isolation of strains, which still holds a dominant place. It is most difficult to draw conclusions where egg medium is employed on account of the complexity of interacting factors.

Recently we were able to obtain in luxuriant growth a strain of blastomyces from a blastomycotic lesion of the skin, on glycerophosphate medium without peptone, suggesting its value in this connection. It is not possible to speak with finality here, owing to the well-known diversity of type presented by organisms from this disease, while its rarity contributes further to the difficulty of obtaining adequate data.

In recent papers, Ayers, Mudge and Rupp<sup>3</sup> have pointed out the advantage of using washed agar, especially in the preparation of milk powder agar. They believe that the washing removes the calcium and magnesium salts from the agar, preventing their precipitation during sterilization, a most objectionable factor in their work.

They have employed distilled water as well as NaCl and N/10 of HCl for the purpose, claiming better results for the latter. Obviously the solvent action of the glycerophosphates for calcium and magnesium salts might be used to advantage in this process, not only as regards the extent to which these salts might be washed out, but also the time consumed in the process. That, of course, would be a matter for experiment to determine.

These authors also found the milk counts higher and the colonies much larger with washed agar. They are inclined to believe that the calcium and magnesium salts are responsible for the bacterial inhibition shown with unwashed agar, although they do not consider this proved. If this is not the case, or when it is desired to make use of the calcium and magnesium salts, it would seem that the glycerophosphates could be employed to advantage. Opportunity, at least, is presented for studying the effect of the calcium and magnesium ions on the growth and isolation of various bacteria.

<sup>3</sup> Jour. Bacteriol., 1920, 5, p. 589.

In connection with milk work another field of usefulness suggests itself. The ordinary methods for the precipitation of casein include the precipitation of the calcium and magnesium salts, which, of course, could be excluded with glycerophosphates.

The tendency to substitute Sorensen's phosphate ( $\text{Na}_2\text{HPO}_4$  with 2 molecules of water of crystallization instead of its normal of 12 molecules) for NaCl in mediums has developed on account of its supposedly stimulating effect on the development of the pneumococcus. This is merely another example of the employment of one or another of the phosphates as growth factors in a more intelligent way than has hitherto been the case.

#### SUMMARY

Disodium glycerophosphate is a solvent for calcium and magnesium and perhaps other salts, and when used in proper concentration prevents much of the objectionable precipitation of phosphates on the alkaline side of neutrality.

This property suggests its employment in culture mediums, in the washing of agar, in the precipitation of casein, and for the study of the effect of the calcium and magnesium ions on the growth of various organisms. The value of the glycerophosphates as food substances is under consideration.

The fact that their ionization constants are substantially the same as those of the nonglycerinated phosphates makes possible this substitution for these salts as buffers. They should be decidedly superior to the latter as buffers, owing to their stability in the lower alkaline ranges where, for example, an initial  $P_H$  of 8 has been maintained in broth after autoclaving.

## A STABLE SINGLE BUFFER SOLUTION

P<sub>H</sub> 1 TO P<sub>H</sub> 12

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It has already been pointed out by one of us (Acree)<sup>1</sup> that there are obvious advantages to be derived from the use of buffer mixtures covering a wide P<sub>H</sub> range, especially if they can be stabilized and so selected as to form a practically continuous, as well as smooth, curve. We know of no single solution that has been recommended so far that embraces these requisites.

Accordingly, we have constructed such a mixture, having components whose dissociation constants are so graded that when the titration curve of one ends the next begins (chart 1). In point of fact, the curves overlap somewhat, involving a condition that will be discussed in detail (chart 2).

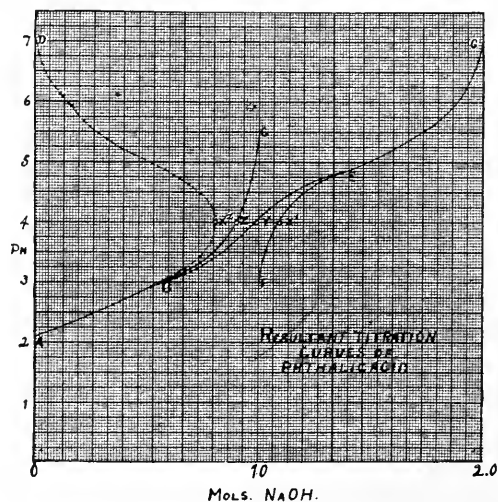
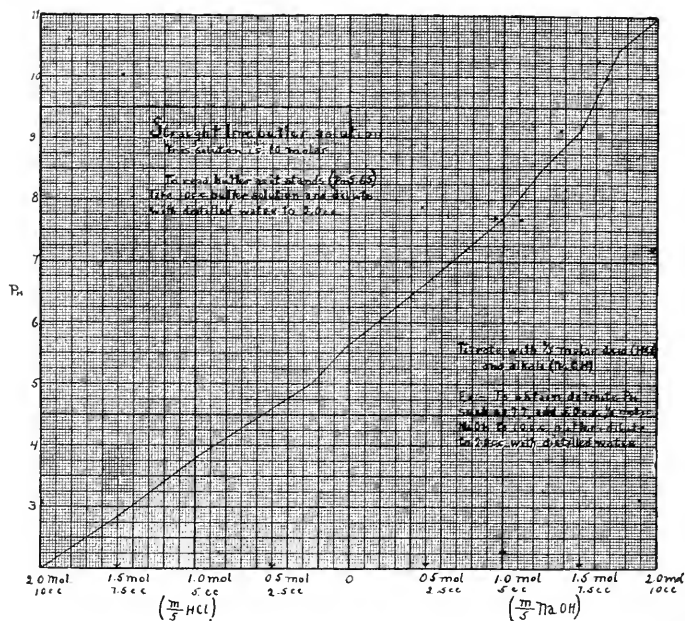
The components, as employed in the buffer solution, are: (1) 1 molecule of KH<sub>2</sub>PO<sub>4</sub>, with a K<sub>a</sub> of  $1.1 \times 10^{-2}$ ; (2)  $\frac{5}{8}$  mol. of sodium formate with a K<sub>a</sub> of  $2 \times 10^{-4}$ ; (3)  $\frac{3}{8}$  mol. of sodium acetate with a K<sub>a</sub> of  $2 \times 10^{-5}$ ; (4) the second group of K<sub>2</sub>HPO<sub>4</sub> with a K<sub>a</sub> of  $2 \times 10^{-7}$ ; (5) 1 mol. of sodium phenol sulphonate with a K<sub>a</sub> of approximately  $10^{-10}$ ; (6) M/200 thymol to saturation (for H<sub>2</sub>O, 0.08) with an approximate K<sub>a</sub> of  $0.5 \times 10^{-10}$ ; (7) the third group of H<sub>3</sub>PO<sub>4</sub> with a K<sub>a</sub> of  $10^{-12}$ .

The ionization constants of sodium phenol sulphonate and thymol have not been measured as yet with the hydrogen electrode, so we are not in possession of all the separate titration curves for the various components. This phase of the subject will be completed shortly, and is not essential for this presentation, as the curve as shown has been developed with the hydrogen electrode and repeatedly checked, both electrometrically and colorimetrically. Points throughout the entire length of the curve were made at intervals of M/8 of alkali or acid, which corresponds to about 0.3 P<sub>H</sub>.

Thymol, used for its well-known antiseptic properties, will probably take the sixth position in the series, based on its close relation chemically

Received for publication Feb. 1, 1921.

<sup>1</sup> Jour. Bacteriol., 1920, 5, p. 491.



to phenol, whose  $K_a$  is known. The solution was developed to fill a practical need; and, by analogy with work done on the resultant curves of other polybasic acids, we have reason to feel confidence in its straight line feature.

In this connection we wish to refer to our study of phthalic acid, a dibasic acid (chart 2). Curves ABC and FEG represent, respectively, titration curves for Na acid phthalate and disodium phthalate if they could be plotted separately, i. e., as representing the complete neutralization of one H before the other has been attacked. Overlapping, however, occurs, eventuating in the resultant curve BYE. Its position has been determined by calculations developed from formulae in papers now in press. The hydrogen electrode has confirmed experimentally the accuracy of the formulation. It is of interest, however, that its position can be located graphically, as shown in chart 2, in the following manner:

Any point of  $P_H$  on FEG is selected, and a measure of the alkali taken from this point back to the ordinate from which the curve starts, as in  $X^1$  on FEG to ordinate at 1. The identical distance is measured from a similar point (one having the same  $P_H$  on the curve ABC forward, and the resulting point will be a point on the resultant curve BE. Assume it is at Y. When this same distance is measured backward horizontally from curve ABC, the curve ABD is developed. From the point  $X^2$  on ABD, forward to Z on ABC represents the amount of the mono-acid salt that has been transformed to disodium salt, and twice this amount ( $X^2Z$ ), that is,  $X^2Y$  is the molar concentration of alkali required to form the corresponding concentration of dibasic salt  $X^2Z$ . It therefore follows that the abscissa values  $OX^2$  plus  $X^2Y$  give the total molar concentration of alkali which has been added to give these amounts of mono- and dibasic salts and the resulting  $P_H$  values, or  $C_H$ . By finding a number of such points Y for  $P_H$  values, we can easily plot the resultant curve ABYEG, giving the relation between the  $P_H$  value and the molar equivalents of alkali added to the phthalic acid.

1. ABC, total curve for sodium acid phthalate formed
2.  $ABX^2$ , curve for NaH salt at any moment
3. ABYEG, resultant curve
4.  $Z^1$ , free phthalic acid

<sup>2</sup> For convenience we have indicated the vertical ordinate by 0 and the ordinate corresponding to one mol. of alkali by 1.

5.  $1X^1$ , disodium phthalate
6.  $ZX^2$ , transformed mono-acid salt

The distance from point X on ABD back to line O, starting point of curve ABC, represents the amount of untransformed mono-sodium salt—Na acid phthalate in this case. Z1 is the amount of free phthalic acid still unneutralized. From this curve we can quantitate the various components of the reaction products found when a dibasic acid is neutralized by alkali. Not only can the individual titration curves be compounded into a resultant curve, but it seems probable that this in turn can be analyzed back into the separate titration curves for each acid or basic group present in unit or fractional equivalents. It may be possible, therefore, to identify quantitatively bases and acids generated in culture mediums of known composition.

Returning to the curve for the single buffer solution: To obtain any desired  $P_H$  it is only necessary to locate the point on the curve that is intercepted by the desired  $P_H$  value, and read off the amount of M/5 HCl or NaOH necessary to produce this  $P_H$ . For example, to obtain a  $P_H$  of 7.7, add 5 c c of M/5 NaOH to 10 c c buffer and dilute to 20 c c with distilled  $H_2O$ . The indicator covering this range is then added.

For convenience we are testing out the feasibility of adding one or two indicator tablets, which are nothing more than sugar of milk, compressed in tablet form and impregnated with a definite quantity of the dye. In the same way, it is often convenient to add tablets of buffers to mediums and biologic fluids. Such measures will receive more detailed treatment at a subsequent time.



## GONOCOCCUS TYPES 2

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In a previous paper<sup>1</sup> it is shown that absorption experiments threw 85 strains of gonococci (1-85) into 6 distinct heterologous types. The agglutinins produced by strains of one type could not be absorbed by strains of another. In 5 the agglutinins were always bound by strains belonging to the same type: that is, the strains that absorbed the agglutinins of one absorbed as well the agglutinins of another. The antigen complex of the strains forming a type seemed to have a similar constitution, and when injected into animals stimulated the production of like agglutinins. Strains forming type 2, however, varied in their agglutinogenic and absorptive capacity, for, while a number of strains bound the agglutinins produced by some strains completely and constantly, the binding capacity in others was more or less limited and variable, and in a few entirely absent. On closer study the 36 strains of this type grouped themselves into 4 fairly distinct and characteristic subtypes, the *a*, *b*, *c*, and *d* races: strains 42-64 form the *a*, 66 and 67 the *b*, 65, 69-74 the *c*, and 68, 75-78, the *d* race. The members of these 4 races will also be referred to as the *a*, *b*, *c*, and *d* strains. The methods used here were the same as in the first paper, and what was said there in regard to them applies here also.

Strain 42 stimulated the formation of agglutinins the affinity of which was limited to the *a* strains. Table 1 gives the absorption results of the serum produced by injections with this strain. One c.c. of a 1:100 dilution of this serum was absorbed by the growth of a large slant each of type 2 strains, and then tested for agglutinins with the immunizing strain. Each *a* strain removed the agglutinins completely, but the *b* strains and the 2 available *d* strains did not. So marked was the avidity of the *a* strains for these agglutinins that even the much larger amount present in 1 c.c. of 1:20 dilutions was altogether bound by 8 of the *a* strains, and only 2 left small traces. The *b* and *d* strains on the other hand failed to extract any in a tenfold, a 1:200, dilution.

Received for publication Feb. 14, 1921.

<sup>1</sup> Hermanies: J. Infect. Dis., 1921, 28, p. 133.

At the time of these tests the *c* and some of the *d* strains were not at hand. Meanwhile, the serum deteriorated, lost its potency and its specificity to such a degree that absorption tests gave unsatisfactory results. Another rabbit was immunized with the same strain. But the serum produced was of a rather low titer. Table 2 gives the results. Again, of the six *a* strains employed, all deprived the serum of its agglutinins, the five *c* and the *d* 67 removed small amounts compared with the other *d* and the control. This rabbit's serum seemed slightly to differ from the previous one in that some of its agglutinins had an affinity for strains of the other races. They apparently were of different nature and present only in relatively small amounts. The bulk of the agglutinins produced by this second rabbit on stimulation with strain 42 could not be absorbed by the *c* and *d* strains although the *a* strains bound them completely. The absorbing strains evidently had an antigen constituent in common with strain 42, and since it differentiated them from the other strains of type 2, and characterized them as members of the *a* race, it was called the *a* agglutininogen, and its antibodies the *a* agglutinins. The latter failed to combine with the members of the other races, because these strains were deficient in the *a* constituent or had it only in small amounts.

The results were different when a serum was produced by injections with *a* 63. A 1:100 dilution was used in table 3. After absorption by the growth of one large slant of strains of type 2, the serum was tested with strain 63. The *a* strains, the *b* 66, and the *c* 65 extracted the agglutinins completely. *B* 67 and the other *c* strains usually left traces unbound. Their absorptive capacities varied: sometimes they absorbed altogether and sometimes they left small amounts. Three of the *d* strains were able to remove moderate amounts of these agglutinins, two failed entirely.

In table 4 a 1:25 dilution was treated in the same way. Strains 65 and 66, that in the previous higher dilution deprived the serum of all the agglutinins, were no longer able to absorb them completely. Only traces remained unbound by strain 65, while strains 66, 73 and 74 left moderate, and 70-72 large, amounts unabsorbed. The absorption by strains 67 and 76 was so limited that enough agglutinins were left in a 1:800 dilution to cause a partial agglutination of strain 63. When this serum was diluted 1:50 and the amount of the agglutinins reduced to half, *c* 65 extracted them completely, *b* 66 and *c* 73 left traces, and the other *c* strains left small and moderate amounts. *B* 67, and the *d* strain

TABLE 1

STRAIN 42, SERUM 1 C C OF 1:100 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 42

Gonococcal Strains	Serum Dilutions			Immunologic Races of Type 2
	1:200	1:800	1:1600	
42, 47, 55-58 .....	0	0	0	a
49, 60, 61, 64 .....	0	0	0	a
66 .....	+++	+++	+	b
67 .....	+++	+++	+++	b
76, 75 .....	+++	+++	++	d
Control .....	+++	+++	++	

In this and the succeeding tables the degree of agglutination is indicated by the number of plus signs, +++ being complete, ++ moderate, + partial but definite; 0 means absence of agglutination.

In the final arrangement of the tables strains giving identical results were treated collectively.

The results of the more decisive dilutions only were recorded in these tables.

TABLE 2

STRAIN 42, SERUM 1 C C OF 1:50 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 42

Gonococcal Strains	Serum Dilutions			Immunologic Races of Type 2
	1:100	1:200	1:400	
43-46, 50-52 .....	0	0	0	a
69-74 .....	+++	+++	0	c
76 .....	+++	+++	0	d
75 .....	+++	+++	+	d
Control .....	+++	+++	+	

TABLE 3

STRAIN 63, SERUM 1 C C OF 1:100 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 63

Gonococcal Strains	Serum Dilutions				Immunologic Races of Type 2
	1:200	1:400	1:800	1:1600	
42-64 .....	0	0	0	0	a
66 .....	0	0	0	0	b
67 .....	++	0	0	0	b
65* .....	0	0	0	0	c
69-74 .....	++	0	0	0	c
68, 75, 76 .....	+++	++	0	0	d
77, 78 .....	+++	+++	+++	+++	d
Control .....	+++	+++	+++	+++	

\* In the first paper strain 65 by mistake was placed in the a instead of the c race.

76 again left the largest amounts unabsorbed. In a dilution of 1:200 the absorption by *d* 68, 75, and 76 was rather limited, by 77 and 78 absent. The *c* strains removed the agglutinins completely, while *b* 67 left traces unbound.

These absorption tests with varying dilutions of the serum produced by immunization with strain 63 thus revealed a marked difference in the binding capacity of type 2 strains for the agglutinins induced with this *a* strain. The agglutininogen that gave rise to this type of agglutinins formed a relatively large and constant constituent in the *a* strains. It must have differed from the *a* agglutininogen, since it was present in the *b* and *c* strains, and even in some of the *d* strains. In 77 and 78 it either was entirely absent or formed such a small compotent in the antigen complex as to have no binding ability for the corresponding agglutinins. The prevalence of this agglutininogen in most of the strains of this type justifies one in considering it a characteristic of the entire type. It was called the *x* agglutininogen and its antibodies the *x* agglutinins.

These absorption experiments furthermore separated strains 42-64 from the other strains of type 2. Strain 42 induced the *a* agglutinins, the affinity of which was limited to the *a* strains, because they alone had the *a* agglutininogen. The *x* agglutinins, incited by the *x* agglutininogen of strain 63, were also absorbed in much larger amounts by the *a* strains. They had, however, a wider range of action and could combine with the majority of the other strains, since the antigen constituent stimulating their production was present in them, though in smaller and variable amounts, and very likely in a somewhat altered configuration. The *a* strains thus revealed the presence of two large constituents in their antigen complex, the *a* and *x* agglutininogens, which enabled them to absorb both types of agglutinins. The members of the other races, though deficient in the *a* agglutininogen, had the *x* agglutininogen as a constituent in their antigen make-up.

The serums of these two strains not only differentiated the *a* strains as members of a distinct and characteristic race, but the *x* agglutinins of strain 63 also distinguished the *d* strains from the two other races as having for these agglutinins either a very limited or an entirely absent binding capacity. The *b* and *c* strains, though varying in their affinities for the *x* agglutinins, could not be separated from one another.

A serum produced by immunization with strain 67 discriminated strains 66 and 67 from strains 65, and 69-74. In table 5 this serum was diluted 1:200, and 1 c.c. of this dilution was absorbed by the growth of

TABLE 4

STRAIN 63, SERUM 1 C C OF 1:25 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 63

Gonococcal Strains	Serum Dilutions						Immunologic Races of Type 2
	1:50	1:100	1:200	1:400	1:800	1:1600	
42, 45, 46, 48-50.....	0	0	0	0	0	0	a
51, 53-57, 64.....	0	0	0	0	0	0	a
66.....	+++	+++	++	0	0	0	b
67.....	+++	+++	+++	++	+	0	b
65.....	++	0	0	0	0	0	c
73, 74.....	++	++	+	0	0	0	c
69-72.....	+++	+++	+++	++	0	0	c
76.....	+++	+++	+++	+++	++	0	d
77, 78.....	+++	+++	+++	+++	+++	++	d
Control.....	+++	+++	+++	+++	+++	++	

TABLE 5

STRAIN 67, SERUM (OLD) 1 C C OF 1:200 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 67

Gonococcal Strains	Serum Dilutions				Immunologic Races of Type 2
	1:400	1:800	1:1600	1:3200	
46, 48, 49, 51.....	+++	+++	+++	++	a
53, 57, 63.....	+++	+++	+++	++	a
56, 45, 47.....	+++	+++	+++	+	a
43, 44, 50, 52.....	+++	++	++	0	b
66.....	+	0	0	0	b
67.....	0	0	0	0	b
71, 74.....	+++	+++	++	0	c
69, 70, 72, 73.....	+++	+++	+++	+	c
75, 76.....	+++	+++	++	+	d
Control.....	+++	+++	+++	+++	

TABLE 6

STRAIN 67, SERUM (OLD) 1 C C OF 1:400 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 67

Gonococcal Strains	Serum Dilutions					Immunologic Types
	1:800	1:1600	1:3200	1:6400	1:12800	
47, 49, 50, 52, 55, 60.....	+++	++	++	+	0	2a
51, 52, 56-58, 63, 64.....	+++	+	+	0	0	2a
66.....	+	0	0	0	0	2b
67.....	0	0	0	0	0	2b
75.....	++	++	+	0	0	2d
76.....	+++	+++	++	+	0	2d
2, 6, 10.....	+++	+++	++	++	0	1
1, 3, 4, 7-9.....	+++	+++	+++	++	+	1
14, 23-29.....	+++	+++	+++	++	+	1
82, 83.....	+++	+++	++	+	0	4
84.....	+++	+++	++	+	0	5
85.....	+++	+++	++	++	0	6
Control.....	+++	+++	+++	++	+	

# J. HERMANIES

one large slant of each of the strains of type 2. When tested for the presence of agglutinins with the immunizing strain only the two *b* strains (66 and 67) removed the agglutinins; the other strains generally failed. Diluted 1:400, and treated like the previous dilution, the results obtained were similar. They are recorded in table 6. Some of the *a* and *d* strains appeared to have extracted part of the agglutinins, others left them fully intact, as did the strains of the other types.

Additional absorptions in dilutions of 1:100, 1:200, and 1:250 were made. With strain 67 the absorption was always complete; strain 66 either bound the agglutinins entirely or left only traces. Some of the *a* strains at one time would deprive the serum of considerable quantities of agglutinins, at another not remove any, even in higher dilutions. Generally their absorptive ability was changeable and limited, or wholly absent. When a 1:200 dilution, after absorption, was tested for the presence of agglutinins with strain 66, the results in the main agreed with those of strain 67 (table 7).

TABLE 7  
STRAIN 67, SERUM (OLD) 1 CC OF 1:200 DILUTIONS ABSORBED BY THE GROWTH OF ONE  
LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 66

Gonococcal Strains	Serum Dilutions				Immunologic Races of Type 2
	1:400	1:800	1:1600	1:3200	
43, 44, 57.....	+++	+++	+++	+	a
47-50, 56.....	+++	+++	++	+	a
52, 53, 46.....	+++	+++	++	0	a
59, 63.....	+++	++	0	0	a
66, 67.....	0	0	0	0	b
71, 73.....	+++	+++	++	0	c
69, 70, 72, 74.....	+++	+++	++	+	c
68.....	+++	++	0	0	d
77.....	+++	+++	++	+	d
Control.....	+++	++	++	++	

Strain 67 thus stimulated the formation of agglutinins the affinity of which was more or less restricted to strains 66 and 67. Since the constituent that incited these agglutinins characterized the members of the *b* race, it was called the *b* agglutininogen and its specific antibodies the *b* agglutinins. Of the other strains the majority were either altogether in want of this constituent or had it in such small quantity as not to combine with perceptible amounts, and if they did combine, this capacity was only passing.

Entirely different facts emerged, when a 1:100 dilution after absorption was tested for the presence of agglutinins with the *a* strain 47. Most of the *a*, *b*, and *c* strains absorbed the agglutinins for this strain completely, and only a few left small amounts unbound. The *d* strains

again either entirely failed or extracted slight amounts. None of the heterologous strains revealed binding capacity.

When a 1:100 dilution, after absorption by strains of type 2, was tested with the *c* strain 71, the *a*, *b*, and *c* strains again absorbed the agglutinins completely. Of the two *d* strains used, 76 absorbed but 77 failed.

The fact that the *a*, *b*, and *c* strains were able to remove the agglutinins for strains 47 and 71 proved that strain 67 had given rise to another type of agglutinins, a type which agglutinated these strains and was absorbed by them. Strain 67 thus stimulated the production of two varieties of agglutinins, the specific or race, and the more general or type, agglutinins. The latter, and the *x* agglutinins of strain 63 were apparently of similar constitution, and were actuated by a compotent analogous to the *x* agglutinin in strain 63. Strain 67 had such a constituent. It was able to absorb the *x* agglutinins of strain 63 in moderate amounts. The lesser and quite limited binding capacity was explained as due to smaller amounts of the *x* entity in this strain. These more general agglutinins no doubt were incited by it. They differed, however, from the *x* agglutinins of strain 63 in that they were absorbed in nearly the same amounts by the *a*, *b*, and *c* strains, while the latter were bound in much larger quantities by the *a* than by the *b* and *c* strains. The members of the last two races besides disclosed a more varied binding ability for the *x* agglutinins of strain 63 than for those of strain 67. It looks as though the *x* agglutinin of strain 67 had a different configuration from the one of strain 63. In *b* 66 and *c* 65 this compotent was more like that in the *a* strains, for they combined with the *x* agglutinins of strain 63 nearly as well as did the *a* strains themselves. Only in the lower dilutions they failed, while in the higher the binding was always complete. Their affinity for these agglutinins was certainly greater than that of the other *b* and *c* strains. The *c* strains generally bound larger amounts of the *x* agglutinins of strain 63 than did the *b* strain 67. Their *x* agglutinin differed from the one of the *a* strains, but had not changed so extensively as that of strain 67, and thus seemed to have an intermediate position. The *x* agglutinin of the *a* strains appeared more alien than the same constituent of the *c* strains to the *x* agglutinins of strain 67. Both races nevertheless removed equal amounts of these agglutinins. Apparently it was present in larger quantities in the *a* than in the *c* strains. These facts justify the assumption that the differences in the avidity for the *x* agglutinins of strain 63

were not solely due to varied amounts of the *x* agglutinin in these races, but somewhat to differences in the structure of this antigen constituent.

The bulk of the *b* agglutinogens in the two *b* strains did not coincide. Strain 67 contained relatively larger amounts of the *b* constituent, since it always was able to bind the *b* agglutinins completely, while strain 66 at times left traces unabsorbed. And the *x* constituent, as already mentioned, was, while similar, not identical in the two strains. The relative proportion of these two constituents was subject to quite marked and sudden changes, as was shown about half a year later with another rabbit. The serum produced with strain 67, was of approximately the same titer as the previous one. When 1 c.c. of a 1:25 dilution was absorbed by strains of type 2, the *a* and *c* strains were able to bind the agglutinins partially (table 9). Now either the *x* agglutinin which strain 67 had in common with the *a* and *c* races had in the meanwhile increased and stimulated a larger production of the *x* agglutinins, or that particular rabbit responded more to the stimulation of the *x* than the *b* component. This serum, no doubt, had larger amounts of the *x* agglutinins, since with it in a much higher dilution there was very little absorption of the agglutinins by the *a* and *c* strains (table 5). In strain 66 the relative proportion of the two components (the *b* and *x* agglutinogens) had swung decidedly in favor of the *x* constituent. It absorbed the *b* agglutinins with difficulty. The *b* agglutinin had meanwhile receded to such an extent that its absorptive capacity for the *b* agglutinins was much reduced. This was confirmed by the fact that, when after absorption by type 2 strains it was used in testing for the presence of agglutinins, most of the *a* and *c* strains extracted the agglutinins for it (table 10). These same strains were unable to absorb the agglutinins of the older serum for strain 66 (table 7). The *b* agglutinins remaining after absorption by these strains were sufficient to induce clumping of strain 66, because it possessed enough of the *b* agglutinin. With the new serum after such an absorption there was no agglutination. Apparently the *b* agglutinin had so receded that the comparatively small amounts of the *b* agglutinins in a dilution of 1:200 were no longer able to exert their action on this strain. However, the *b* agglutinin had not disappeared entirely, the strain still retaining some *b* characteristics. Thus when the serum was only diluted 1:100, strains 66 and 67 absorbed completely, the *a* and *c* strains generally left small amounts unbound.



TABLE 8

STRAIN 67, SERUM (OLD) 1 Cc OF 1:100 DILUTIONS ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 47

Gonococcal Strains	Serum Dilutions				Immunologic Types
	1:200	1:400	1:800	1:1600	
42, 48, 50-53.....	0	0	0	0	2a
56, 57, 63, 64.....	0	0	0	0	2a
54, 60.....	++	0	0	0	2a
66.....	+	0	0	0	2b
67.....	0	0	0	0	2b
69, 70, 72, 74.....	0	0	0	0	2c
71.....	++	0	0	0	2c
73.....	+++	+	0	0	2c
75.....	+++	+++	+	0	2d
76.....	++	++	++	+	2d
77.....	+++	+++	+++	++	2d
1, 5, 7, 38-40.....	+++	+++	++	+	1
3, 4, 6, 9-12, 14.....	+++	+++	+++	++	1
16, 17, 19, 22, 41.....	+++	+++	+++	++	1
81.....	+++	+++	+++	+	3
83.....	+++	+++	+++	0	4
84.....	+++	+++	+++	+	5
85.....	+++	+++	+++	++	6
Control.....	+++	+++	+++	++	

TABLE 9

STRAIN 67 SERUM (NEW) 1 Cc OF 1:25 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 67

Gonococcal Strains	Serum Dilutions					Immunologic Races of Type 2
	1:50	1:100	1:200	1:400	1:800	
54, 57, 64.....	+++	+++	+++	+	0	a
46, 48, 51, 63.....	+++	+++	++	+	0	a
42, 45, 50, 53.....	+++	+++	++	0	0	a
66.....	+++	+++	+	0	0	b
67.....	0	0	0	0	0	b
70, 72-74.....	+++	+++	+	0	0	c
65.....	+++	+++	++	0	0	c
69, 71.....	+++	+++	+++	+	0	c
68.....	+++	++	+	0	0	d
76-78.....	+++	+++	+++	++	+	d
Control.....	+++	+++	+++	+++	++	

TABLE 10

STRAIN 67, SERUM (NEW) 1 Cc OF 1:200 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 66

Gonococcal Strains	Serum Dilutions				Immunologic Races of Type 2
	1:400	1:800	1:1600	1:3200	
42, 46, 49, 50, 51.....	0	0	0	0	a
54, 57, 59, 63, 64.....	0	0	0	0	a
48.....	+	0	0	0	a
53.....	++	+	0	0	a
66-67.....	0	0	0	0	b
69-72, 74, 65.....	0	0	0	0	c
73.....	+	0	0	0	c
68.....	0	0	0	0	d
76.....	+++	+++	0	0	d
77, 78.....	+++	+++	+	0	d
Control.....	+++	+++	++	+	

When the same dilution after absorption was tested for the presence of agglutinins with the *c* strain 71, the *a*, *b*, and *c* strains absorbed the agglutinins completely, showing that the *x* agglutinins could be handled by these strains. The small *b* component could still absorb some of the *b* agglutinins; if their amounts were large it absorbed enough to induce flocculation of strain 66. In the presence of smaller amounts the absorption was not sufficient for its agglutination.

The members of the *a*, *b*, *c* races were linked to each other by the *x* agglutinins, and their relationship in the type was thus established directly. The *d* strains on the other hand showed little or no affinity for these agglutinins, and their relation in type 2 remained doubtful. Strains 68, 75, and 76 generally bound small amounts though their absorptive capacity varied much. Strain 76 once absorbed the *x* agglutinins of strain 67 for strain 71, and strain 68 absorbed them completely for strain 66 and nearly so for 71. At times the absorption was quite marked, at other times it was absent. Strains 77 and 78 generally failed, and the slight occasional absorption would not justify placing them in this type.

TABLE 11

STRAIN 69, SERUM 1 C C OF 1:100 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 69

Gonococcal Strains	Serum Dilutions					Immunologic Types
	1:200	1:400	1:800	1:1600	1:3200	
48-51, 53, 54.....	+++	+++	+++	++	+	2a
42, 44, 45, 47, 63.....	+++	+++	+++	++	+	2a
46, 64.....	+++	+++	+++	++	0	2a
66, 67.....	+++	+++	+++	++	+	2b
65.....	+++	+++	+++	++	0	2c
69-74.....	0	0	0	0	0	2c
76-78.....	++	0	0	0	0	2d
3, 16, 41.....	+++	+++	+++	+	0	1
1, 2, 4, 5, 7, 10.....	+++	+++	+++	++	+	1
11, 14, 20-22, 39.....	+++	+++	+++	++	+	1
32, 34, 36, 40.....	+++	+++	+++	++	+	1
79-81.....	+++	+++	+++	++	+	3
83.....	+++	+++	+++	++	+	4
84.....	+++	+++	+++	++	+	5
85.....	+++	+++	+++	++	+	6
Control.....	+++	+++	+++	++	+	

Absorption tests with a serum produced by the *c* strain 69 established them, however, as a distinct race of type 2. Table 11 gives the results. One c.c. of 1:100 dilution of this serum was absorbed by the growth of one large slant of the following strains, and then tested for agglutinins with strain 69. None of the *a* and *b* strains of types 2 were able to absorb any. The *c* strains, however, with one exception absorbed

them completely, and the *d* strains left only traces. The strains of the other types bound none of the agglutinins. In a dilution of 1:200 the *a* and *b* strains again failed, but strain 67 absorbed partially. *C* 65, failing in the previous lower dilution, now completely bound the reduced amount of the agglutinins. The *d* strains removed all the agglutinins. When a 1:100 dilution after absorption was tested with strain 76, every one of the *c* strains absorbed the agglutinins, the two *d* strains again leaving traces. *B* 67 was able to bind fairly large amounts of agglutinins for this strain (table 12). Note that even a few of the *a* strains appear to have extracted some. In the last two tables *d* 77 was used in testing for the presence of agglutinins after absorption by the various strains. The results were clear-cut. When the serum was diluted 1:100, as in table 13, all the *c* and *d* strains absorbed the agglutinins completely, but the *a* and *b* strains left them wholly intact. Even in a 1:400 dilution, as in table 14, they failed to bind any agglutinins, in which they did not differ from strains of the heterologous types.

These absorption results show that strain 69 produced agglutinins which could not be bound by any *a* strains. *B* 66 was never able to absorb, though *b* 67 absorbed partially for 69 in a 1:200 dilution, and nearly completely for 76 in a 1:100 dilution. The agglutinins were without difficulty bound by all *d* strains. They must have had a compotent similar in constitution to that in the *c* strains, and present in relatively the same proportions. This same compotent was either entirely absent or present in exceeding small amounts in most *a* and *b* strains. To some extent, however, it entered into the composition of strain 67, since on two occasions it showed a tendency to bind these agglutinins. The quite extensive though limited absorptive capacity of the *c* strains for the *x* agglutinins of the serum induced by 63 and 67 showed that all had the *x* agglutininogen as a constituent in their antigen complex. In strain 69, however, this compotent did not stimulate the formation of agglutinins. Several attempts were made to determine their presence. When a 1:100 dilution after absorption was tested with the *a* and *b* strains the controls failed to agglutinate. Since these strains were agglutinated with difficulty and only in low dilutions, the amount of the *x* agglutinins, if at all present, was limited indeed. It was the *c* agglutininogen that the *d* strains shared in common with the *c* strains. This enabled them to absorb the *c* agglutinins and indirectly linked them to this type.

TABLE 12  
STRAIN 69, SERUM 1 C C OF 1:100 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE  
SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 76

Gonococcal Strains	Serum Dilutions					Immunologic Races of Type 2
	1:200	1:400	1:1600	1:3200	1:6400	
46, 50, 63.....	+++	+++	+++	+++	++	a
49, 51, 53.....	+++	+++	++	++	+	a
44, 48, 54, 57.....	+++	+++	++	++	0	a
45, 64.....	+++	+++	+	0	0	a
66.....	+++	+++	+++	++	++	b
67.....	++	+	0	0	0	b
65, 69-74.....	0	0	0	0	0	c
77, 78.....	++	0	0	0	0	d
Control.....	+++	+++	+++	+++	++	

TABLE 13  
STRAIN 69, SERUM 1 C C OF 1:100 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE  
SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 77

Gonococcal Strains	Serum Dilutions			Immunologic Types
	1:200	1:3200	1:6400	
42, 46, 49, 50.....	+++	+++	+++	2a
51, 53, 57, 63.....	+++	+++	+++	2a
45, 44, 48, 54, 64.....	+++	+++	+++	2a
66, 67.....	+++	+++	+++	2b
65, 69-74.....	0	0	0	2c
68, 76-78.....	0	0	0	2d
7, 16.....	+++	+++	+++	1
15.....	+++	+++	+++	1
84.....	+++	+++	++	4
Control.....	+++	+++	+++	

The heavy type indicates that after absorption by these strains there was partial agglutination of the testing strain in a 1:12,800 dilution.

TABLE 14  
STRAIN 69, SERUM 1 C C OF 1:400 DILUTION ABSORBED BY THE GROWTH OF ONE LARGE  
SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 77

Gonococcal Strains	Serum Dilutions			Immunologic Types
	1:1600	1:3200	1:6400	
42, 44-46, 48-51.....	+++	+++	+++	2a
53, 54, 57, 64.....	+++	+++	+++	2a
63.....	+++	+++	0	2a
66, 67.....	+++	+++	+++	2b
22.....	+++	+++	0	1
1, 4, 5, 10-12, 41.....	+++	+++	+++	1
14-16, 19, 42.....	+++	+++	+++	1
3, 7, 34, 36, 39, 40.....	+++	+++	+++	1
81.....	+++	+++	+++	3
83.....	+++	+++	+++	4
84.....	+++	+++	+++	5
85.....	+++	+++	+++	6
Control.....	+++	+++	+++	

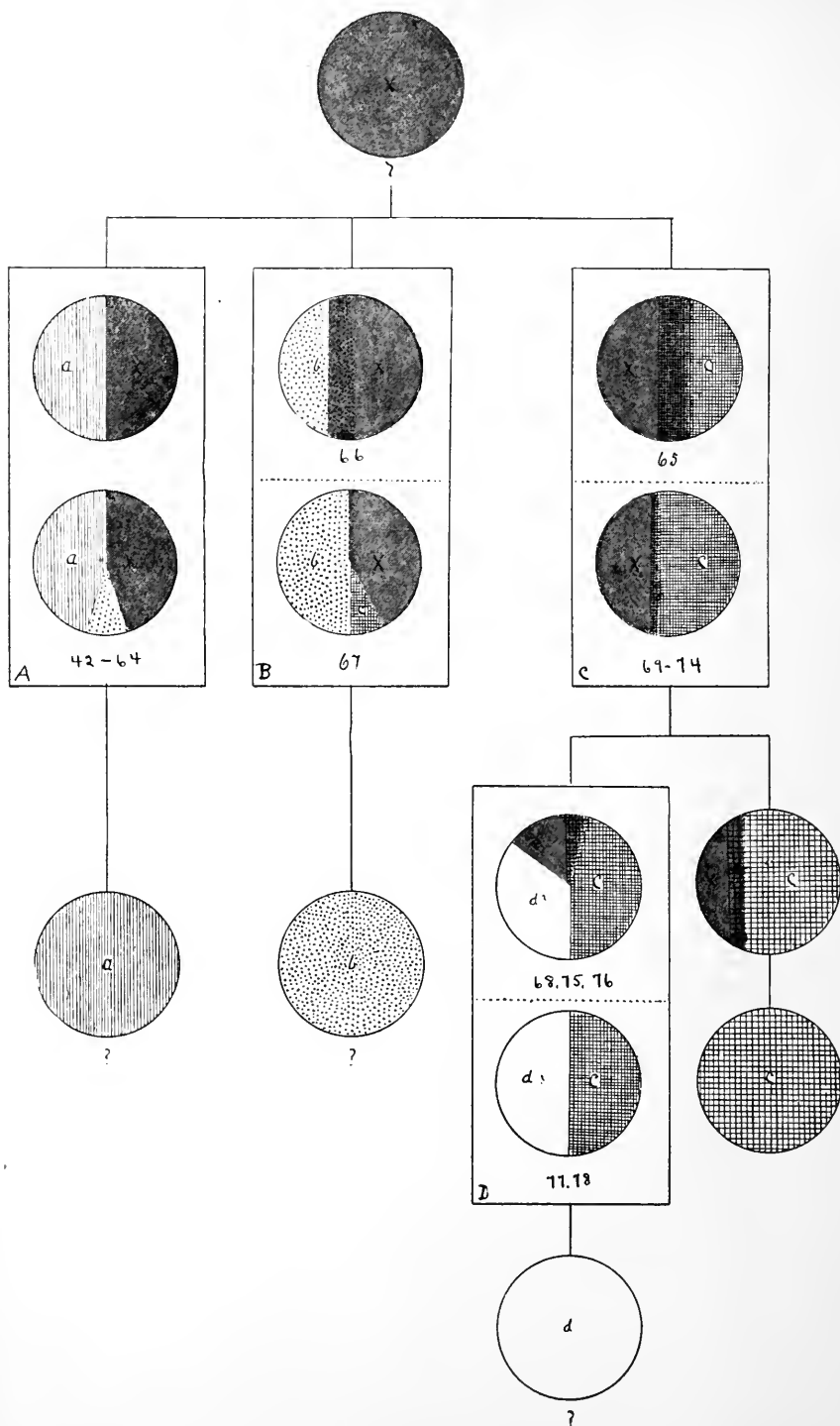
## DISCUSSION

The antigen complexes of the 4 races of type 2, their interrelationships, and their probable path of evolution are shown graphically in the genealogic table:

The circles included in the *A*, *B*, *C*, and *D* squares represent the antigen of the *a*, *b*, *c*, and *d* races. The *a* strains brought to light two large and permanent compotents—the *a* and *x* agglutinogens—the relative proportions of which, while fairly constant, were nevertheless, subject to slight variations, variations more pronounced in some strains than in others. In strain 42 the *a* agglutinin stimulated the formation of the *a* agglutinins, the affinity of which was limited to the *a* strains, since the *a* moiety was their specific characteristic. The representatives of the other races failed to combine with them, because they lacked this entity. In strain 63 the *x* agglutinin was active and incited the *x* agglutinin which had a wider but varied range of action. Their avidity for the *a* strains was marked, more restricted and variable for the *b* and *c* strains, quite limited or absent for the *d* strains. This variation in the binding capacity can be explained as due to differences in amounts and in the constitution of the *x* constituent in the strains of the 4 races, and was tentatively indicated by the diversity in size and shading of this compotent in the diagram. Both strains had the 2 compotents, the *a* and *x* agglutinogens being alternately active and passive.

The antigen of the 2 members of the *b* race also had 2 main constituents, the *b* and *x* agglutinogens, and when strain 67 was used for immunization both actuated their respective agglutinins. The affinity of the specific race agglutinins was restricted to the *b* strains, the more general *x* agglutinins being bound with comparative ease and in approximate equal amounts by the *a*, *b*, and *c* strains. In this they differed from the *x* agglutinins of strain 63, which were absorbed in varying amounts by these strains.

These two *x* agglutinins were of similar but not identical constitution and differed in their molecular arrangement. They offer proof that the *x* agglutinin of strain 67 had a different configuration than the same moiety in strain 63. This is in accord with the assumption that this compotent had a slightly different and varying constitution in the several races. Thus the variable binding capacity of the several races and even of the individual strains was at least partially due to the differences in the configuration of the *x* agglutinogens and not merely to variations in amounts.



Genealogy of four races of type 2.

The *c* strains had the *x* agglutinin, since they were able to bind both varieties of the *x* agglutinins. The quantity and configuration apparently differed from the same entity in the other races and varied even in the individual strains as indicated by their somewhat fluctuating and unequal binding capacity. They had their specific race constituent, the *c* agglutinin. In strain 69 it alone was active and led to the formation of the *c* agglutinins. The latter with one exception had no affinity for the *a* and *b* strains. They were, however, absorbed constantly in large amounts by the *c* and *d* strains. The *d* strains thus shared this constituent in common with the *c* strains. It entered, however, into the make-up of the antigen of strain 67, since this strain was occasionally able to combine with fairly large amounts of the *c* agglutinins.

*D* strains differed from *c* strains in the slighter avidity for *c* agglutinins, and in the limited or absent binding capacity for both *x* agglutinins. They were represented as composed of two families which differed from each other in that the antigen complex of the one had a small amount of the *x* agglutinin while the antigen complex of the other had none. They evidently had another undetermined constituent the nature of which was not revealed—no serum having been produced. Notwithstanding, this constituent was assumed and regarded as specific for these strains, and was called the *d* agglutinin. The *c* agglutinin may, however, have undergone alteration of structure and thus despite their presence in larger amounts in these strains, the latter had a lesser affinity for the *c* agglutinins than the *c* strains proper. This possibility is visualized in the last two figures on the right. In that case they should be regarded rather as variants of the *c* race, the agglutinin of which either had receded still farther or was lost entirely.

The *x* agglutinin, excepting the two *d* strains, was a common constituent of all the races of this type, and is no doubt primary and oldest. Evidently at one time it was the sole competent and is as such represented in the hypothetical type from which these several races of type 2 differentiated themselves. Owing to the inherent tendency of living matter to vary, some strains succeeded, either by new combinations or by altering the configuration of some of the *x* agglutinogens and by changing them into certain ways, in elaborating new structures. Thus, if a certain modification proved to be advantageous to the species the strains exhibiting this variation were favored. Starting from this advantage, the variation continued in that particular direction until a

new constituent was evolved. It may have been in an attempt to overcome and evade the antibodies developed by the host against its specific antigen that certain strains differentiated this new component. Their advantage once revealed, they became exalted and increased in amounts, the old meanwhile receding. Some strains may have acquired this through gradual and progressive alteration. In others the change may have been abrupt. Again in some strains this variation and differentiation led to the formation of the *a* agglutinin, in others it resulted in the *b* and *c* moieties. At least these 3 and possibly 4 variations had definitely established themselves in the 36 strains of this type. The primary *x* antigen not only receded, but also underwent slight modification which was along different lines in several races. In the 22 strains forming the *a* race, the *a* and *x* constituents appeared to be fairly balanced. Evidently all the gonococci in these strains had both constituents. The newly acquired racial character had stabilized and firmly established itself. The temporary fluctuations in the individual strains apparently were due to transient changes in the relative proportion of these 2 components. The same conditions seem to prevail in the majority of the *b* and *c* strains. The more marked and sudden changes in *b* 66 and in *c* 65 call for some other explanation. Thus in 66 the *b* moiety must have receded to very small proportions, since its binding capacity for the *b* agglutinins became very limited, and only when they were present in large amounts was it able to combine with sufficient quantities to agglutinate the strain. Apparently this strain was composed of two varieties of organisms. Some had acquired the *b* agglutinin and consequently had both the racial and the type agglutinogens in their antigen complex. Others were deficient in the specific *b* constituent, and their antigen had merely the *x* component, thus approaching the assumed primary hypothetical type composition from which these races supposedly developed. If by chance the bulk of the culture consisted of the latter, the strain extracted little or no racial agglutinins. The degree of absorption to a certain extent thus depended on the relative proportion of the two types of organism having the *xb* and the *x* structure. At an earlier date these two types were fairly balanced, though subject to daily variations, one in excess of the other. Thus one day the strain was able to bind the *b* agglutinins of strain 67 completely and on the next day left traces. Later the organisms with the *x* constituents decidedly dominated. Its binding ability for the *b* agglutinins was very limited. It shows how by still



farther reduction of the organisms having the racial *xb* antigen complex this strain may have reverted to the hypothetical *x* type. It affords a good example of how pure change is a factor in the development of the immunologic types from preexisting races, or the reverse.

The fact that strain 67 on several occasions was able to bind fairly large amounts of the *c* agglutinins, though generally failing to absorb any of them, can best be explained on the same supposition. No doubt there were some gonococci that had the *c* agglutigen in their antigen complex. If thus it chanced that in a certain culture they were present in great numbers, the strain was able to absorb large amounts of the *c* agglutinins. Some of the *a* strains had cocci that had the *b* agglutigen in their antigen composition, because occasionally some of them were able to absorb fairly large amounts of the *b* agglutinins of strain 67. Even the *c* strain 65 must have had organisms with the simple *x* antigen composition, since once it failed to absorb any of the *c* agglutinins.

The three *d* strains that still had the *x* agglutigen in their antigen complex were quite erratic in their ability to bind the *x* agglutinins. Generally they absorbed little but sometimes a strain was able to bind large quantities. It apparently was composed of two types of organisms, one having the *xc*, the other the *cd* antigen combination, and the natural fluctuation in the relative proportion of these two organisms explained their varying absorptive abilities. The *d* strains 77 and 78 no doubt had lost the *xc* component entirely.

The hypothetical antigen composition below the 4 races indicates how by a farther shifting and elimination of the *x* constituent 4 distinct types can be evolved. Strains having these antigen compositions would have no relation whatsoever with each other and would constitute 4 separate and distinct immunologic types. It shows how immunologic species may eventually be formed from races by the gradual elimination of an old and common constituent that linked these races into a single type. That such a final elimination is possible was shown by strains 77 and 78 of the *d* race. Had it not been for the *c* agglutinins which they were able to bind they would have to be excluded from type 2, and would then constitute a new type. Judging from analogy the 6 distinct and separate gonococcic types discussed in the previous paper may have sometime been merely races of one or two types. In the course of evolution the common connecting bond was eliminated and the races became new species. As the process of variation is still going on, and will continue as long as their living conditions are secured, there is no

limit to farther differentiation. A single clear-cut type may by molecular rearrangement acquire new antigen constituents and split into several races. Finally, by elimination these may differentiate into species.

This marked lability and variation in quantity and constitution of the various compotents constituting the antigen complexes of the races of type 2 show that a strain is potentially able to revert to the original type or differentiate into variants definite and characteristic enough to constitute fairly distinct races. Not all the compotents present in an antigen stimulate the formation of agglutinins; in some strains one, and in others a different agglutininogen actuates its specific agglutinins. All this complicates the grouping of strains having such variable and complex antigen composition, necessitating a large number of serums for the establishment of their interrelationships within a single type. Thus type 2 is unique and distinct from the other 5 types, no strains of other types being able to absorb its agglutinins.

# THE CHANGE IN THE HYDROGEN-ION CONCENTRATION OF VARIOUS MEDIUMS DURING HEATING IN SOFT AND PYREX GLASS TUBES

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An exact knowledge of the reaction of a medium can be gained only from a determination of its hydrogen-ion concentration. It is a well established fact that sterilization of a medium or subjection of certain organic solutions to high temperatures for long periods increases their acidity. The presence in such mediums of the so-called "buffers," however, prevents rapid changes in the hydrogen-ion concentration, even if the titratable acidity is considerably altered.

In order to determine accurately the thermal death point of heat resistant spores at high temperatures it is of utmost importance to control all the factors which influence it. In a previous paper<sup>1</sup> it has been shown that even a slight change in the hydrogen-ion concentration greatly affects the thermal death point. It is necessary, therefore, in the determination of thermal death points to heat under conditions which will maintain a constant hydrogen-ion concentration throughout the entire period.

It is a well-known fact that heating solutions in soft glass tubes causes a certain amount of alkali to go into solution. Russell, Nichols and Stimmel<sup>2</sup> have found that the reaction of typhoid vaccine may vary considerably when held in soft glass containers at room temperature and depends on two factors; the amount of soluble alkali in the glass containers, and the amount of carbon dioxide absorbed from the air. They state that in itself the typhoid bacillus does not change the ordinary reaction of the medium ( $P_H$ -7.4-7.6) in which there is no sugar which is fermented by the typhoid bacillus. If the vaccine, however, is put in soft glass containers, it becomes alkaline.

Received for publication Feb. 14, 1921.

<sup>1</sup> W. D. Bigelow and J. R. Esty: The Thermal Death Point in Relation to Time of typical Thermophilic Organisms, Jour. Infect. Dis., 1920, 27, p. 602.

<sup>2</sup> Military Surgeon, 1920, 47, p. 539.

The following results reported by them are self-explanatory:

1. Vaccine placed in soft glass ampules in 1911.....	9.26
2. Vaccine placed in soft glass ampules in 1911.....	9.70
3. Vaccine placed in hard glass ampules in 1911.....	8.10
4. Vaccine placed in hard glass ampules in 1919.....	7.4
5. Vaccine placed in hard glass ampules in 1919.....	7.6

In a personal communication with K. F. Meyer he states that there is a similar condition in bile which has been held in soft glass containers for some time.

Hard glass tubes have been suggested as a means of controlling this important factor during the heat treatment.

The object of this investigation is to determine the effect on the hydrogen-ion concentration of heating "unbuffered" solutions and solutions rich in "buffers" for long periods and at high temperatures in soft and hard glass tubes. In the article by Bigelow and Esty<sup>1</sup> results are given on the thermal death points of spores of certain thermophilic organisms when heated at different temperatures in food juices. These juices were heated in soft glass tubes and electrometric measurements made at definite intervals to determine the change in the hydrogen-ion concentration during the heating. At the same time determinations were made on the same juices heated for similar periods in hard glass tubes. Experiments were also conducted in which various solutions were heated in soft and hard glass tubes and the effect of the heating on the hydrogen-ion concentration noted.

A few results are also given in this paper to show the difference in the time necessary to destroy a definite suspension of spores in corn juice when heated in hard and soft glass tubes.

#### PREPARATION OF TUBES PRIOR TO HEATING

The soft and hard glass tubes used in this work were 7 mm. (inside diameter) by 250 mm. long with a 1 mm. thickness of wall specially designed for our thermal death point work. They were held in a weak hydrochloric acid solution over night and then rinsed several times with tap water. The tubes were then refilled with distilled water and autoclaved at 15 pounds for 30 minutes after which they were rinsed 3 times with distilled water and drained. The dry tubes were wrapped in packages of 10 each with heavy wrapping paper and sterilized at 160 C. for 3 hours. These sterilized tubes were then ready to receive the juice, and the different tests were made. An

examination of the tables in this paper shows that there is no rapid change in the hydrogen-ion concentration of solutions in soft and hard glass tubes until they have been heated.

#### METHOD

A series of soft and hard glass tubes each containing 5 c.c. of the solution to be tested was sealed and heated at different temperatures. At definite intervals tubes were removed from the constant temperature bath and the hydrogen-ion concentration determined. Duplicate samples were run in every case and in some instances 3 or 4 samples were used and the average obtained. The food juices heated in this work were those pressed from canned corn, peas, spinach, string beans, beets, sweet potatoes and pumpkin. In the case of sweet potatoes and pumpkin, an equal volume of distilled water was added and the resulting juice pressed as in the other juices. It was impossible to obtain the juice from these undiluted canned products due to their heavy consistency. Different mixtures of disodium acid phosphate and potassium diacid phosphate, freshly distilled water, physiologic salt solution, and weak solutions of hydrochloric acid and sodium hydroxide were also heated in these tubes, and the hydrogen-ion concentration determined at definite intervals. The electrometric method was employed exclusively in these determinations and controls used throughout.

#### CHANGE IN THE HYDROGEN-ION CONCENTRATION OF UNBUFFERED SOLUTIONS DURING HEATING IN SOFT AND HARD GLASS TUBES

The change in the hydrogen-ion concentration of unbuffered aqueous solutions during heating in soft glass tubes was determined by heating freshly distilled water, physiologic salt solution and solutions of hydrochloric acid and sodium hydroxide at 120 C. for 20 and 30 minutes. The effect of heating was noted on unheated solutions and also on the same solutions in soft glass tubes given a preliminary heating of 100 C. for 5 minutes in order to expel the  $\text{CO}_2$  in solution. Physiologic salt solution and solutions of hydrochloric acid and sodium hydroxide were heated at 120 C. for 15 and 30 minutes in hard glass tubes to determine the change in the hydrogen-ion concentration during heating.

Table 1 shows that the hydrogen-ion concentration of these unbuffered aqueous solutions changes enormously when heated in soft

and hard glass tubes. There is great variation in the  $P_H$  values of individual determinations in the unheated as well as in the heated solutions. This may be due partly to a variation in the individual tubes, which in the case of soft glass contain some soluble alkali and in the case of hard glass a certain amount of acid. Since there are no buffer salts present in these solutions, even a small amount of acid or alkali would greatly affect the hydrogen-ion concentration. The results, however, show an increase in acidity as the heating is prolonged in hard glass tubes while an increase in alkalinity is observed in soft glass tubes.

TABLE 1  
EFFECT OF HEATING UNBUFFERED SOLUTIONS IN SOFT GLASS

Treatment	P <sub>H</sub> Value of Solutions				
	NaCl 0.85%	Distilled H <sub>2</sub> O	HCl N/100,000	NaOH N/1,000	NaOH N/100,000
0					
1. Heated in sealed tube 2. 120 C. for 20 min.	6.85	7.28	6.97	9.92 9.38	8.54
Heated in sealed tube 3. 120 C. for 30 min.	9.54	10.07	10.00 10.13	9.40 9.30	9.16
Heated in sealed tube 4. 100 C. for 5 min.	9.54 9.60	10.01 10.04	9.86 9.88	9.32 9.62	8.91
Heated in open tube 5. 100 C. for 5 min.	9.24	8.70	9.67 9.74		
Treated as 4 then sealed 5. heated 120 C. for 30 min.	9.61 9.61		9.98 10.01		

Effect of Heating Unbuffered Solutions in Hard Glass

Treatment	P <sub>H</sub> Value of Solutions				
	NaCl 0.85%	HCl N/1,000	HCl N/100,000	NaOH N/1,000	NaOH N/100,000
0					
	6.96	3.02 3.04	6.50 7.56	10.03 9.20	8.02 7.53
120 C. - 15 min.	5.84 5.02	3.03 3.02	7.19 6.52	9.15 8.73	7.56 7.39
120 C. - 30 min.	5.19 4.48	3.00 2.98	7.00 7.10	8.86 8.28	6.13 5.90

These results are given to demonstrate the marked change in the hydrogen-ion concentration of unbuffered aqueous solutions before heating and after heating at 120 C. for varying times. It definitely shows the inability and impracticability of using unbuffered solutions in the determination of thermal death points when it is desired to maintain a constant hydrogen-ion concentration throughout the procedure.

CHANGE IN THE HYDROGEN-ION CONCENTRATION OF PHOSPHATE  
MIXTURES HEATED AT 120 IN SOFT AND  
PYREX GLASS TUBES

Mixtures of the secondary and primary phosphates were used in this test and prepared in 200 cc amounts as follows:

M/5 $\text{Na}_2\text{HPO}_4$	M/5 $\text{KH}_2\text{PO}_4$	Approximate $\text{P}_\text{H}$
190 cc	10 cc	8.0
120 cc	80 cc	7.0
20 cc	180 cc	6.0
10 cc	190 cc	5.5
2 cc	198 cc	5.0
0 cc	200 cc	4.5

The actual  $\text{P}_\text{H}$  values of these mixtures are shown in table 2.

Table 2 gives the results obtained showing the change in the hydrogen-ion concentration of the different mixtures after exposure at 120 C. for times varying from 5 to 75 minutes, since that was in excess of the time necessary to destroy the most resistant spores in that medium of a hydrogen-ion concentration requiring the longest exposure for complete sterilization. These figures indicate that when these mixtures are heated in hard glass tubes the hydrogen-ion concentration remains constant during the time required for the complete destruction of highly heat resistant spores, whereas when the same mixture is heated in soft glass tubes, a decided increase in alkalinity is observed. This lowering in the hydrogen-ion concentration is much more marked in an alkaline solution, as shown by a difference in the  $\text{P}_\text{H}$  values of 1.14 for a solution of  $\text{P}_\text{H}$  7.89. In the neutral solution the change is negligible during the entire heating period, the average  $\text{P}_\text{H}$  readings showing only a difference of 0.06. As the more acid mixtures are heated the change again becomes apparent. The differences in the  $\text{P}_\text{H}$  of the unheated acid solutions and those heated for 75 minutes become greater as the more acid mixtures are used. A change of 0.23 is noted in the original solution of  $\text{P}_\text{H}$  5.86, 0.45 in the original solution of 4.91 and 0.65 in the original solution of 4.51.

There is a slight change in the hydrogen-ion concentration of the more acid mixtures heated in hard glass tubes, a change in the  $\text{P}_\text{H}$  values of 0.04 and 0.05 being noted in the original solutions of  $\text{P}_\text{H}$  values 4.91 and 4.46, respectively. This change takes place within the first 10 minutes heating and is hardly sufficient to consider since it lies within the range of possible experimental error in the test. Apparently the buffer salts in the other mixtures control the small

amount of acidity produced by heating and prevent a change in the hydrogen-ion concentration.

Since the increase in acidity produced by heating the mixtures irrespective of the glass tubes is not appreciable, the amount of soluble alkali in the soft glass tubes is unaffected except by the action of the buffer salts and the progressive lowering in the hydrogen-ion concentration results. This same effect was noted at temperatures of 100 C. and above. Although no work was done below boiling, it is safe to assume that a similar condition would exist during an equivalent heating. These results below show that hard glass tubes must be used in heating these phosphate mixtures if a constant hydrogen-ion concentration is to be maintained during a long period.

TABLE 2  
EFFECT OF HEATING PHOSPHATE MIXTURES

Container	PH Value of Solutions Heated at 120 C. for											
	0 Min.	5 Min.	10 Min.	15 Min.	20 Min.	25 Min.	30 Min.	40 Min.	45 Min.	50 Min.	60 Min.	75 Min.
Soft glass	7.89		8.02		8.00		8.25		8.24		8.50	9.02
Hard glass	7.88	7.85	7.97		8.05	7.89	7.88	7.87	8.60	7.86	8.98	9.03
			7.88		7.86					7.86	7.87	7.87
Soft glass	6.94		6.93				6.96		7.0		6.97	6.97
Hard glass	6.94	6.95	6.94	6.94	6.95	6.95	6.98	6.95	7.0	6.95	7.16	7.02
			6.95				6.95				6.94	6.94
Soft glass	5.86	5.88		5.90	5.91	5.95	5.94	5.92		5.97	5.96	6.09
Hard glass	5.88	5.88	5.86		5.87		5.87		5.87		5.87	5.88
Soft glass	4.91	5.10	5.10	5.05	5.02	5.10	5.04	5.20			5.23	5.36
Hard glass	4.91		4.88		4.87		4.86	4.86			4.86	4.87
Soft glass	4.51	4.69	4.60	4.72		4.78	4.80	4.91		5.08	5.02	5.16
Hard glass	4.46		4.39		4.39		4.39		4.39		4.40	4.41

EFFECT ON THE HYDROGEN-ION CONCENTRATION OF CORN AND PEA JUICES HEATED AT 100 C. IN SOFT AND HARD GLASS TUBES

Table 3 shows the change in the hydrogen-ion concentration of corn and pea juices when heated for varying lengths of time at 100 C. in soft and hard glass tubes. Results of duplicate and triplicate samples show a slight variation in individual determinations, but a comparison of the average values demonstrates the effect produced during a prolonged heating.

Heating in soft glass tubes does not produce a uniform lowering or raising of the hydrogen-ion concentration of either of these juices, but a similar change is taking place during the same periods. A slight increase in alkalinity is observed within the first few hours, due to the



presence of the soluble alkali in the glass. The hydrogen-ion concentration remains fairly constant for another period during which interval the acid produced by heating these juices is sufficient to neutralize any additional alkali that dissolves out. Prolonged heating increases the amount of acid to such an extent that there is an excess of hydrogen-ions to hydroxyl-ions, and hence an increase in the hydrogen-ion concentration results. During the heating period (24 hours), however, this is not sufficient to differ greatly from the original unheated juices. If heated for an indefinite period, it is assumed that the hydrogen-ion concentration would be greatly affected.

TABLE 3  
EFFECT OF HEATING CANNED CORN AND PEA JUICES

	Container	PH Value of Solutions Heated at 100 C. for							
		0 Hrs.	2 Hrs.	6 Hrs.	10 Hrs.	12 Hrs.	16 Hrs.	20 Hrs.	24 Hrs.
Corn juice	Soft glass	5.98					6.15	5.99	5.97
		5.97	5.94	6.07	6.06	6.05	6.07	6.05	5.96
		6.00	6.05	6.07	6.06	6.05	6.14	5.98	6.06
		5.98	5.99	6.07	6.06	6.05	6.12	6.01	6.00
	Hard glass	5.98		5.83	5.70	5.68	5.55		5.46
		5.98	5.85	5.84	5.74	5.70	5.56	5.50	5.46
		5.97	5.85	5.82	5.68	5.70	5.59	5.52	5.46
		5.98	5.85	5.83	5.73	5.69	5.57	5.51	5.46
Pea juice	Soft glass	5.58		5.64	5.64	5.69		5.55	5.57
		5.58		5.63	5.63	5.65		5.62	5.63
		5.58		5.64	5.64	5.67		5.58	5.60
	Hard glass	5.58		5.50	5.38	5.41		5.30	5.26
		5.58		5.50	5.44	5.41		5.28	5.27
		5.58		5.50	5.41	5.41		5.29	5.27

On the other hand, there is a progressive increase in the acidity of these juices when heated in hard glass tubes which results in a steady increase in the hydrogen-ion concentration. In this respect heating these juices in hard glass tubes gives a different effect than heating a highly buffered solution, such as the phosphate mixtures discussed in a previous section.

Although the hydrogen-ion concentration of these juices does not remain constant during heating in either hard or soft glass tubes, yet it is more constant when the latter are used, and it is believed that results obtained in thermal deathpoint determinations in mediums of definite P<sub>H</sub> value are more accurate when soft glass tubes are used than when hard glass tubes are used. The same relationship exists at temperatures above 100 C. for periods sufficient to sterilize solutions containing very resistant spores.

EFFECT ON THE HYDROGEN-ION CONCENTRATION OF VEGETABLE  
JUICES WHEN HEATED IN SOFT AND HARD  
GLASS TUBES AT 120 C.

Table 4 shows that the hydrogen-ion concentration is not appreciably altered when the juices pressed from canned corn, peas, spinach, string beans, beets, sweet potatoes and pumpkin are heated in soft glass tubes at 120 C. for different times. Heating these juices for similar periods

TABLE 4  
EFFECT OF HEATING CANNED VEGETABLE JUICES

Juice	Container	P <sub>H</sub> Value of Solutions Heated at 120 C. for					
		0 Min.	10 Min.	20 Min.	30 Min.	45 Min.	60 Min.
Corn	Soft glass	5.98	6.06	6.05	6.01	6.00	6.00
	Hard glass	5.98	5.83	5.69	5.57	5.52	5.46
Pea	Soft glass	5.58	5.60 5.51		5.59		5.56 5.60
	Hard glass	5.58	5.50	5.53	5.49	5.46	5.43
Spinach	Soft glass						4.61 4.61
	Hard glass	4.60	4.61 4.59	4.58	4.61 4.58		4.62 4.56
String bean	Soft glass		4.49 4.46	4.52		4.49	
	Hard glass	4.45 4.46	4.50 4.44	4.49 4.44	4.50 4.42	4.50 4.41	4.50 4.40
Sweet potato	Soft glass		4.42 4.55	4.50	4.50 4.48		4.45 4.43
	Hard glass	4.42	4.52 4.40	4.46 4.40	4.51 4.38	4.36	4.43 4.34
Beet	Soft glass					4.26 4.27	4.26 4.27
	Hard glass	4.27	4.28 4.24	4.27 4.24	4.27 4.24	4.22	4.27 4.20
Pumpkin	Soft glass	3.97 3.98	4.02 4.00	4.00	4.00		4.00 3.99
	Hard glass	3.97 3.98	3.97	3.99	4.00	3.98	3.98

in hard glass tubes increases their hydrogen-ion concentration, thus making it impossible to determine the thermal death point in these juices at a constant hydrogen-ion concentration. The juice pressed from canned pumpkin is not altered to the extent that the other juices are when heated in hard glass, the results showing a fairly constant hydrogen-ion concentration throughout. The hydrogen-ion concentration of these juices, however, is higher than that of the raw or canned product since they have been reesterilized after they were pressed. A greater change would be observed in the hard glass tubes if the hydrogen-ion concentration of the original juice was more nearly that of the

canned product. These results warrant the use of soft glass tubes in the determination of the thermal death points of suspensions heated in the vegetable juices named.

The results thus far reported in this paper on the effect of heating food juices have been obtained on juices that have been pressed from sterile canned foods and resterilized in the autoclave. The initial hydrogen-ion concentration of these juices was considerably higher than that of the raw unprocessed product. It is interesting to note that the same general change in the hydrogen-ion concentration takes place in the raw food juice much lower in acidity than that of the cooked product.

To determine this effect, a sample of corn was cut from the cob, brine consisting of 6% sugar and 1.25% salt added, and the juice squeezed through a straining cloth. The brine content is about the normal amount used in the canning of corn. This juice was distributed in a series of hard and soft glass tubes and heated at 115 and 120 C. for definite intervals from 10 minutes at 120 C. to 120 minutes at 115 C. The color of the product was also observed. The results obtained on the hydrogen-ion concentration are given in table 5.

TABLE 5  
EFFECT OF HEATING RAW CORN JUICE

	P <sub>H</sub> Value of Corn Juice Heated at 115 C. for							Heated at 120 C. for			
	0 Min.	30 Min.	45 Min.	60 Min.	75 Min.	90 Min.	120 Min.	0 Min.	10 Min.	20 Min.	30 Min.
Soft glass		7.20	6.64		6.55	6.56	6.50		6.88		
	6.85	7.15	6.68	6.56	6.66	6.53	6.48	6.84	6.94	6.72	6.82
	6.84	6.83	6.70	6.58	6.62	6.54	6.54	6.87	6.99	6.78	6.80
	6.87	7.14	6.85	6.54	6.54	6.56	6.54	6.85	7.01	6.65	6.76
Average	6.85	7.08	6.72	6.56	6.59	6.55	6.52	6.85	6.96	6.72	6.79
Hard glass	6.86	6.60		6.30		6.15	6.11	6.86	6.45	6.18	6.11

The same general change takes place as in the other juices. Heating in soft glass tubes at 120 up to 30 minutes affects the hydrogen-ion concentration of this juice appreciably. The average P<sub>H</sub> values show an initial lowering in the hydrogen-ion concentration and then an increase to value remaining fairly constant. There is some variation in individual determinations. Using hard glass tubes, on the other hand, the acidity greatly increases as the time is prolonged. The color of the juice darkens as the cooking is continued, due to the caramelization of the sugar.

THE THERMAL DEATH POINT OF SPORES IN CORN JUICE HEATED IN  
SOFT GLASS AND HARD GLASS TUBES AT  
100, 115, AND 120 C.

The thermal death point of spores in corn juice was determined according to the method described by Bigelow and Esty.<sup>1</sup> Table 6 shows the comparative results obtained on the time necessary to destroy a known concentration of spores when heated in corn juice in soft and hard glass tubes at 100, 115 and 120 C. It shows conclusively that the time is materially shorter in every case when hard glass tubes are used, due to the progressive increase in acidity obtained during the heating period.

TABLE 6  
THERMAL DEATH POINT OF DIFFERENT CONCENTRATIONS OF SPORES (CULTURE NO. 1503)  
IN CANNED CORN JUICE AT SPECIFIED TEMPERATURES AND RESULTING  
CHANGE IN PH VALUE

Container	Spores per C c	Heated at	Time Required to Destroy		Initial P <sub>H</sub>	Final P <sub>H</sub>
			+	—		
Soft glass.....	22,000	100 C.	24 hr.	25 hr.	6.00	6.00
Hard glass.....	22,000	100 C.	20 hr.	21 hr.	6.00	5.62
Soft glass.....	2,200	100 C.	22 hr.	23 hr.	6.00	6.00
Hard glass.....	2,200	100 C.	19 hr.	20 hr.	6.00	5.62
Soft glass.....	14,000	115 C.	105 min.	110 min.	6.13	6.12
Hard glass.....	14,000	115 C.	80 min.	85 min.	6.13	5.70
Soft glass.....	1,400	115 C.	90 min.	95 min.	6.13	6.12
Hard glass.....	1,400	115 C.	75 min.	80 min.	6.13	5.70
Soft glass.....	22,000	120 C.	30 min.	32 min.	6.13	6.10
Hard glass.....	22,000	120 C.	27 min.	30 min.	6.13	5.70
Soft glass.....	2,200	120 C.	25 min.	27 min.	6.13	6.10
Hard glass.....	2,200	120 C.	22 min.	24 min.	6.13	5.70

The results obtained in soft glass tubes are at a nearly constant hydrogen-ion concentration throughout and represent more accurately the actual time required to destroy at a definite hydrogen-ion concentration.

This table also shows the effect of the initial concentration of spores on the time necessary to destroy them. The results tabulated in the column indicating the time required to destroy spores give the last time at which growth occurred represented by a + sign and the first time at which growth failed to occur showing the destruction of all the spores represented by a — sign. The results shown in the column headed final P<sub>H</sub> give the P<sub>H</sub> value of the corn juice heated for a period sufficient to destroy the known suspension of spores, the time corresponding to that given in the right hand column headed "Time Required to Destroy Spores."

## CONCLUSIONS

Heating unbuffered solutions in soft glass tubes greatly affects the hydrogen-ion concentration.

Heating mixtures of  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$  in hard glass tubes does not affect the hydrogen-ion concentration during the heating, while prolonged heating in soft glass tubes dissolves out alkali in excess of the amount which can be controlled by the buffer salts. Heating an alkaline solution of this mixture in soft glass causes a greater lowering in the hydrogen-ion concentration than acid solutions. A neutral solution does not change appreciably during heating even in soft glass tubes.

Heating the juices pressed from canned corn, peas, string beans, spinach, beets, sweet potatoes, and pumpkin in soft glass tubes affects the hydrogen-ion concentration less than in hard glass tubes.

A longer time is necessary to destroy the same suspension of spores in corn juice if heated in soft glass tubes than in hard glass tubes.

No general statement can be made regarding the relative merits of hard and soft glass tubes in the determination of the thermal death point. The type of glass to be used for this purpose must be determined for each solution.

In thermal death point determinations the hydrogen-ion concentration of the solution must be known during the entire period of heating.

# CHANGES IN THE ALKALI RESERVE, SUGAR CONCENTRATION, AND LEUKOCYTES OF THE BLOOD IN EXPERIMENTAL INFECTIONS

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A previous study of the changes in the leukocytes and the alkali reserve of the blood with experimental infections in rabbits demonstrated that the intravenous injection of suspensions of living pathogenic bacteria is followed within 1 to 2 hours by a leukopenia and a diminution of the alkali reserve of the whole blood.<sup>1</sup> The rapidity and the degree of lowering of the alkali reserve in these experiments seemed to depend on the pathogenicity of the bacteria used. Following depression of the alkali reserve and the leukopenia there is an 18 to 24 hour interval during which the leukocytes increase rapidly in number, and the alkali reserve returns to, or slightly exceeds, the value originally determined for the blood of the rabbit. When the alkali reserve remains depressed the leukocytosis persists, but later when it approaches or exceeds slightly the normal the number of leukocytes also becomes normal. In experiments in which the infection does not subside with such simple and prompt changes, there are irregular depressions of the alkali reserve and coincident periods of leukocytosis until both remain normal, and there is no further evidence by these examinations of disease in the animal. The suggestion is made, on the basis of these results, that lowering the alkali reserve of the blood sufficiently, or the factors associated with its depression, are concerned with the production of a general leukocytosis.

An excellent summary of the theories advanced to explain generalized leukocytosis was given by Gehring.<sup>2</sup> Following Metschnikoff's studies of the activities of leukocytes and their highly important function in immunity, the value of artificially produced leukocytosis in combating infectious diseases was generally recognized. At first, variations in the number of leukocytes of the blood were thought to occur only in the peripheral vessels, but later this

Received for publication Feb. 16, 1921.

<sup>1</sup> Hirsch: Jour. Infect. Dis., 1921, 28, p. 275.

<sup>2</sup> Ztschr. exper. Path., 1915, 17, p. 161.

was disproved. From the earliest studies, the theories concerning the origin of leukocytosis included the notion of some chemical substance attracting the leukocytes into the circulation from reserve places or stimulating their formation in the hematopoietic tissues. Many experiments *in vitro* demonstrated chemical substances with a positive chemotaxis toward leukocytes but all these are inconclusive as regards a satisfactory explanation of a generalized leukocytosis.

A further step in explaining generalized leukocytosis was made by demonstrating that toxins stimulate the leukocyte-forming tissues such as bone marrow. Other chemical agents, such as a toxin acting on the smooth muscle of the spleen and lymph nodes, mechanically forcing the cells contained into the circulation (lymphocytosis) and stimulation of the hematopoietic tissues by the disintegration products of red blood corpuscles, have been given as causes of leukocytosis. The leukocytosis of hemorrhage was thought to result from some indirect stimulus of the bone marrow, and other substances such as the breakdown products of leukocytes themselves were considered for a time as causing leukocytosis.

The confusion in explaining leukocytosis was increased by observing leukocytosis after the action of certain physical agents on the body, such as massage, heat or cold, trauma, muscular activity, chemical irritation of the skin, psychic stimuli, pregnancy, etc., also leukocytosis with digestion.

Pepper and Miller<sup>3</sup> in studying the leukopenia and subsequent leukocytosis in rabbits after injections of both living and killed typhoid bacilli, could establish no relationship between leukocytosis and the content in the urine of the total nonprotein urea, and allantoin nitrogen, all end-products of nucleic acid metabolism which these authors thought might be correlated with changes in the number of leukocytes of the blood. This briefly summarizes what is at present contained in the literature to explain generalized leukocytosis.

With the changes mentioned in the alkali reserve of the blood, there are without doubt others as yet unsuspected and of a chemical nature, since alterations in H-ion concentration are known to accompany or bring about disturbances in the composition and properties of such an intricate solution of colloids and crystalloids as blood plasma. One of these changes, it was fair to suppose, concerns the dextrose of the blood, and some hint of changes in the amount of glucose in the blood with infections is found in the report of Rohdenberg and Pohlman<sup>4</sup> who consider the hyperglycemia of animals immunized against bacteria an index of the degree of immunization. Elias<sup>5</sup> observed hyperglycemia and glycosuria in rabbits and dogs following the feeding of dilute hydrochloric acid, while even earlier writers, such as Pavy in 1864, Goetz in 1867, and Naunyn in 1868<sup>6</sup> had

<sup>3</sup> J. Infect. Dis., 1916, 19, 694.

<sup>4</sup> Am. J. Med. Sc., 1920, 159, p. 853.

<sup>5</sup> Biochem. Ztschr., 1913, 48, 120.

<sup>6</sup> Kulz, E.: Beiträge zur Lehre vom künstlichen Diabetes, Arch. ges. Physiol., 1881, 24, p. 97.

observed glycosuria following the feeding or injection of phosphoric, lactic, or hydrochloric acids. Underhill,<sup>7</sup> commenting on the effects of acidosis on carbohydrate metabolism, says the introduction of acid into the organism is associated with a disturbance of blood sugar content and is manifested in one direction; namely, blood sugar content is augmented. Other writers have made similar comments.<sup>8</sup>

With these observations as a basis, an experimental study in rabbits was made to determine the effect on the blood sugar concentration of

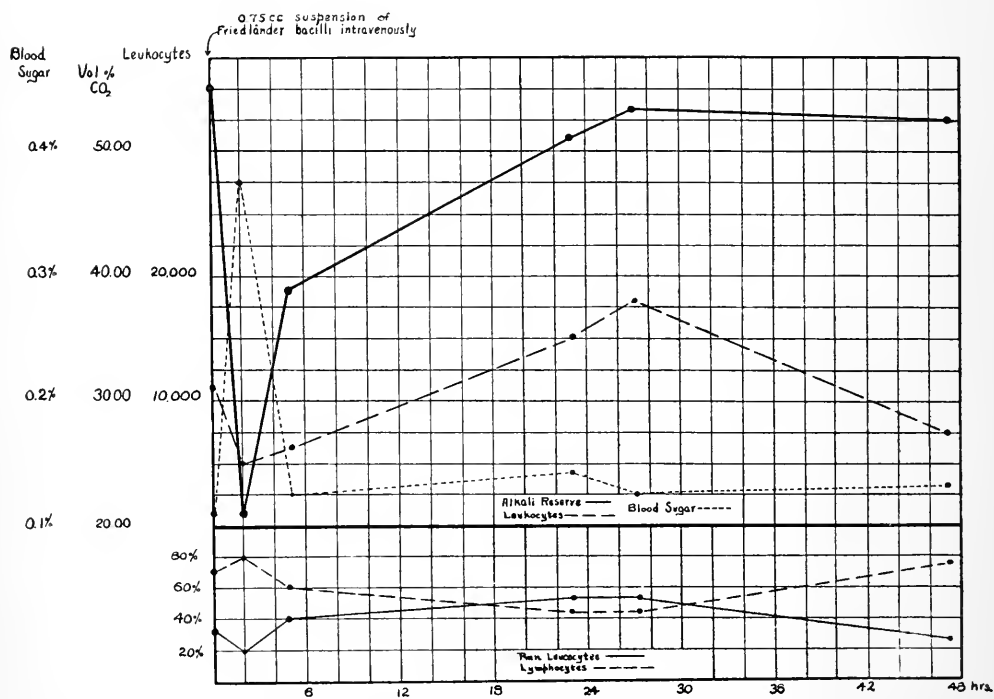


Chart 1.—Effect of injection of living pathogenic bacteria.

intravenous injections of suspensions of living pathogenic bacteria, paralleled by estimations of the alkali reserve of the whole blood, and by determinations of the number of leukocytes. The following bacteria were used: *B. typhosus*, *B. paratyphosus* A, *B. paratyphosus* B, *B. dysenteriae* (Flexner), *B. Friedländer*, *B. coli*, streptococcus hemolyticus, Pneumococcus, and *B. welchii*. The bacteria were grown on

<sup>7</sup> Jour. Biol. Chem., 1916, 25, p. 463.

<sup>8</sup> Mathews, A. P.: Physiological Chemistry, 1915, p. 247.





plain or blood-agar slants in pure culture for 14-48 hours, and varying fractional amounts depending on their pathogenicity in rabbits were injected intravenously in 0.75 to 2.0 c c volumes of sterile normal salt solution. The alkali reserve, the sugar concentration, and the number of leukocytes of the blood were determined before, and at, intervals after the injections, the amount of blood taken from the ear veins for each set of determinations being from 3 to 4 c c. The blood sugar estimations were made according to Folin and Wu<sup>9</sup> with the special tubes recommended, the alkali reserve of the whole blood according to Van Slike and Cullen.<sup>10</sup>

With the initial lowering of the alkali reserve of the blood there is a sudden and transient increase in the concentration of the sugar in the blood which in degree seems to be in proportion with the depression of the alkali reserve. This transient rise in the concentration of the sugar seemingly reaches its maximum with the lowest level to which the alkali reserve of the blood falls and then returns within 2 to 4 hours to the normal concentration even though the alkali reserve remains depressed. The accompanying chart graphically represents this change in an experiment with Friedländer bacilli, and in the table are summarized the results of experiments with other bacteria and those with injections of glucose and acid potassium phosphate solutions. The alkali reserve, the concentration of sugar, and the number of leukocytes were not changed in the blood of rabbits injected intravenously with sterile normal salt solution (10 c c) or subjected only to the handling and bleeding necessary for the experiments.

The injection of acid potassium phosphate solutions depresses the alkali reserve of the blood, and with this there is an increase in the concentration of the sugar in the blood similar to that produced by the injections of bacteria. In other experiments with the injection of these solutions, a leukocyte curve was observed similar to those produced by bacteria.

Having observed depression of the alkali reserve of the blood regularly after injections of pathogenic bacteria in rabbits, attempts were made to prevent this depression by subcutaneous injections of sodium carbonate and bicarbonate solutions. Two equal-sized rabbits received intravenously equal amounts of a typhoid bacillus suspension, one of them subcutaneously 18 c c of a 5% sodium carbonate solution (equiva-

<sup>9</sup> Jour. Biol. Chem., 1919, 38, p. 81. Ibid., Supplement I., 1920, 41, p. 367.

<sup>10</sup> Ibid., 1917, 30, p. 289.

lent to 188 c.c. 0.1 N NaOH) in divided fractions, the other rabbit 8 c.c. of sterile normal salt solution. The alkali reserve of the rabbit receiving the carbonate solution decreased from 67.27 carbon dioxide volumes per cent. to 27.86 at the 25th hour when the animal died. The other rabbit lived, and the leukocyte, alkali reserve, and sugar concentration curves of each are similar to those given for typhoid and other pathogenic bacteria. The depression of the alkali reserve in other rabbits injected with colon and Friedländer bacilli could not be prevented by sodium carbonate solutions given subcutaneously.

Differential leukocyte counts were made of the blood of rabbits before and at intervals after the injection of bacteria. In the rabbits examined and regarded as normal the leukocytes of the blood are distributed approximately as follows: lymphocytes—large 10 to 15%, small 35 to 55%; transitional leukocytes, 0 to 1%; mononuclear leukocytes, 0 to 1%; polymorphonuclear leukocytes—neutrophil, 30 to 50%, eosinophil, 0 to 1%, basophil 1 to 6%.

During the early part of the leukopenia stage the percentage of lymphocytes is somewhat greater than that of the polymorphonuclear leukocytes (chart). In a short time the polymorphonuclear ratio rises so that as the circulating white blood cells increase the percentage of neutrophil leukocytes may rise to 80 or 90. Later the lymphocytes form an increasing proportion of leukocytes until finally the normal ratio is again reached. When the number of leukocytes decreases rapidly with the return to normal of the alkali reserve, the polymorphonuclear leukocytes seem to disappear first, leaving a high percentage of lymphocytes in the blood. In a number of experiments with the injection of acid phosphate solutions the changes in the number and ratio of leukocytes followed curves similar to those obtained with bacteria.

#### COMMENT

An increased concentration of sugar in the blood with acidosis is well known. On this basis, then, the occurrence of hyperglycemia with experimental infections seems to depend on a lowering of the alkali reserve (acidosis), and the degree of hyperglycemia on the extent to which the alkali reserve is depressed. Milroy<sup>11</sup> observed a loss of the alkali reserve of the blood after hemorrhage, but reported no studies of the variations in the concentration of the blood sugar.

<sup>11</sup> Jour. Physiol., 1917, 51, p. 259.

Tatum<sup>12</sup> found in rabbits a lowering of the alkali reserve and a hyperglycemia immediately after severe hemorrhage. In 12 to 24 hours the sugar content of the blood returned to normal, and the alkali reserve returned to, or sometimes exceeded, the value determined as normal. Another interesting observation on the relation of lowered alkali reserve of the blood to hyperglycemia was made by Peters and Geyelin<sup>13</sup> who found that the injection of epinephrin in diabetic patients and in normal human beings was accompanied by a simultaneous diminution of the alkalinity of the blood and a hyperglycemia. They regard decreased alkalinity of the blood as important in producing hyperglycemia of this type. Rohdenberg and Pohlmann<sup>4</sup> make no reference to alkali reserve changes in the blood which presumably resulted from their injections of bacteria.

While the notion that an acidosis accompanies acute infections has been expressed and attempts to correct it have been made by the administration of carbonate solutions, one of the clearest demonstrations of a change in the H-ion concentration of the blood in acute infections has been made recently by Dragstedt.<sup>14</sup> He observed in rabbits infected with hemolytic streptococci a variation in the H-ion concentration of the blood of from  $P_H$  7.65 or 7.75, which is normal, to  $P_H$  7.3 in the infected rabbits shortly before death.

#### CONCLUSIONS

Depression of the alkali reserve of the blood in rabbits by intravenous injections of pathogenic bacteria is accompanied by a transient hyperglycemia, the degree of hyperglycemia apparently depending on the extent of alkali reserve diminution.

Subcutaneous administration of carbonate or bicarbonate solutions does not prevent the acidosis produced by these injections of bacteria.

Injections of acid potassium phosphate solutions depress the alkali reserve of the blood, this lowered alkalinity being associated with a hyperglycemia and by changes in the number of leukocytes similar to those following injections of bacteria.

The concentration of sugar in the blood seems to be independent of the changes in the number of leukocytes.

<sup>12</sup> Jour. Biol. Chem., 1920, 41, 59.

<sup>13</sup> Ibid., 1917, 31, p. 471.

<sup>14</sup> Jour. Infec. Dis., 1920, 27, p. 452.

# BACTERIOLOGIC STUDIES OF THE UPPER RESPIRATORY PASSAGES

## I. HEMOLYTIC STREPTOCOCCI OF THE ADENOIDS

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Numerous bacteriologic investigations of the throat and nasopharynx have been carried out to determine the incidence of dangerous bacteria in the normal as well as in diseased conditions. Most of the previous observations were made from swab cultures of the throat and pharynx, and as it is impossible for this method to reach the recesses of the tonsillar crypts and folds of the adenoids, it is obvious that the results obtained are inaccurate and not a true index of the flora of these regions. In a previous work by Pilot and Davis<sup>1</sup> it was noted that the crypts of 100 pairs of extirpated tonsils contained hemolytic streptococci often in large numbers in 97%, whereas from the swab cultures of the same persons before tonsillectomy the same organisms were recovered in fewer numbers in only 61%. In order to ascertain the extent to which the other lymphoid structures harbor the streptococci a study of the flora of the extirpated adenoids of a similar group of persons was undertaken, together with a smaller series of extirpated tonsils for comparison.

In the present work cultures were made from the adenoids of 103 children. In 25 instances swabs of the nasopharynx were obtained by means of the West tube before adenoidectomy and in the same group both the extirpated tonsils and adenoids were studied. In the remaining 78 cultures were made from the adenoids. The patients were children varying from 5 to 16 years of age who presented adenoids and tonsils of varying degrees of hyperplasia with no evidence of any recent acute inflammation, fever or subjective symptoms of sore throat. The adenoids in most instances were removed by the La Force adenectomy and the tonsils by the Beck tonsillectome under general anesthesia at the Cook County Hospital during the months of April, May and June, 1920.

Received for publication Feb. 16, 1921.

<sup>1</sup> Jour. Infect. Dis., 1919, 24, p. 386.

The adenoids consisted of hyperplastic lymphoid tissue from 1 to 1.5 cm. square and about 1 cm. thick. Structurally many presented from 3 to 6 deep folds; in others fibrous union had taken place between the folds leaving pits not unlike the tonsillar crypts but not quite as deep. Most of the specimens had both folds and crypt-like depressions. Fatty debris and cholesterol crystals were encountered in a few instances, but in none was a purulent exudate seen. The tonsils of the same persons, like the adenoids, were of lymphoid tissue of varying degrees of hyperplasia. Occasionally the crypts contained fatty debris and hard yellow actinomyces-like granules. Such granules were not grossly visible in the adenoids.

THE PERCENTAGE OF HEMOLYTIC STREPTOCOCCI IN CULTURES OF THE NASOPHARYNGEAL SWABS AND ADENOIDS OF THE SAME PERSONS

	Number of Persons	Percentage Positive
Series i.		
Swab cultures .....	25	40
Cultures from depths of adenoids.....	25	60
Series ii.		
Cultures from the surface of the adenoids.....	78	58
Cultures from the depths of the adenoids.....	78	62
Total number of swab and adenoid surfaces from which cultures were taken.....	103	55
Total number of cultures from the adenoid depths....	103	61

All the material was collected separately in sterile gauze and cultures made in 1 to 4 hours after removal. Material for culture was obtained by streaking the epithelial surface of the vegetations with a wire loop and then carefully separating the folds and mouths of the pits with sterile forceps another culture was obtained from the depths. The tonsils were inverted with the capsule outside and incised transversely at right angles to the crypts with a sterile knife and cultures made from the bottom of the crypts. The wire loops were streaked on the surface of blood-agar plates made up of infusion agar titrated to a hydrogen-ion concentration of 7.6 to which human blood was added in proportion of one part of blood to 10 to 15 parts of agar. In addition the same medium in the melted state of 45 C. was inoculated from the same sources and poured into plates. The plates were incubated at 37.5 C. and examined at the end of 24 and 48 hours for hemolytic colonies.

Small, discrete, biconvex, gray-white colonies forming zones of complete hemolysis measuring from 1 to 4 mm. across were noted and isolated in pure culture on blood-agar slants for further study and confirmation,

In the cultures of the nasopharyngeal swabs hemolytic streptococci were present in 10 of 25 instances, or in 40% of the cases. From the same persons the streptococci were present in the depths of the adenoid vegetations in 15, or 60%, demonstrating the inaccuracy of the swab culture. In the remaining 78 cultures made from the surface of the adenoids they occurred in 45, or 58%, as compared with 48, or 62%, positive in the adenoids depths. In both the swab and surface cultures they were relatively few in number, seldom predominant and numerous in only 9 instances. In the depths, however, they were present in decidedly larger numbers, being quite numerous in 18 instances and in pure culture in 3. The foregoing figures were obtained by examination of the poured plates. It is interesting to note that from the streaked blood-agar plates the hemolytic streptococci were observed in only 26% of the cultures of the adenoid surface and in 37% of those of the depths, demonstrating definitely the superiority of the poured plate in the detection of these organisms.

Of the 21 pairs 20, or 95%, revealed hemolytic streptococci in either both or one tonsil. In the adenoids of the same persons these organisms occurred in 15, giving 5 instances in which streptococci were recovered from the tonsils and not from the adenoids. Furthermore, the tonsillar crypts harbor these organisms in greater numbers as compared with the adenoids. The crypt-like structures of the nasopharyngeal vegetations, like the crypts of the tonsils, frequently contain these streptococci in strikingly large numbers, showing the strong tendency of the hemolytic streptococcus to flourish in the deep depressions of the lymphoid tissue of the oro- and naso-pharynx.

The streptococci corresponded to the beta type of Smith and Brown. The narrow, indefinitely hemolytic colonies of the alpha type were present in 20% and were included in the nonhemolytic streptococcus group.

Sixty-five strains of hemolytic streptococci, of which 60 were isolated from the adenoids, were studied in pure culture. All were bile insoluble and hemolytic in the subcultures. In infusion carbohydrate broth they formed a flocculent sediment, while the supernatant fluid usually remained clear. Smears revealed gram-positive cocci in moderately long or often very long chains. Four differential sugars were inoculated—lactose, salicin, mannite and inulin. The medium consisted of infusion broth with 1% carbohydrate and Andrade indicator. Readings were made at the end of 4 and 10 days. All fermented lactose,

all but 3 salicin; 3 fermented mannite, and 2 inulin. Inulin from another source was not fermented by the 2 strains. According to Holman's classification, 59 were streptococcus pyogenes, 2 strep. infrequens, 2 strep. anginosus, and 1 strep. hemolyticus 3. Litmus milk was acidified by all in 7 days, 10 coagulating spontaneously and the remainder on gentle heating.

Eight strains were selected at random and injected intravenously into rabbits. These strains were isolated from the adenoids or the nasopharynx. In 2 instances 2 c c of a serum-broth culture incubated 48 hours were employed and one rabbit died in 24 hours with evidences of a septicemia and beginning arthritis. The second rabbit died in 72 hours and at necropsy had a moderately purulent polyarthritis and vegetations on the aortic valves. Five other rabbits, weighing from 800 to 2,000 gm., were inoculated with a blood-agar slant (incubated 48 hours) suspended in salt solution. One died in 48 hours and revealed petechial hemorrhages on the serous surfaces and many streptococci in the heart blood. Another succumbed on the 13th day from a marked purulent polyarthritis and peri-arthritis, and periostitis of two adjacent ribs. The remaining 3, killed on the 11th day, showed polyarthritis of varying severity. One rabbit which received one-half of blood-agar slant also developed purulent arthritis. The joints involved were chiefly those of the wrists, ankles, knees and phalanges. Five other strains were introduced into white mice in doses of 0.5 c c of a 24-hour serum-broth culture intraperitoneally. Four died within 18 hours and the fifth in 36 hours, and from all the streptococci were recovered from the peritoneal exudate and the heart blood.

#### SUMMARY

Hemolytic streptococci are common in the nasopharynx and nasopharyngeal vegetations. From nasopharyngeal swabs and the surface of the adenoids hemolytic streptococci were recovered in 55%; from the depths between the folds and of the crypt-like depressions of the adenoids of the same persons, in 61% in larger numbers. The excised tonsils of the same patients revealed hemolytic streptococci in still larger numbers in 95%.

These streptococci agree in their morphology, cultural characteristics, fermentation reactions and pathogenicity, and are practically identical with hemolytic streptococci from various human sources.

The adenoids, like the tonsils, are to be considered as common foci harboring hemolytic streptococci.



## BACTERIOLOGIC STUDIES OF THE UPPER RESPIRATORY PASSAGES

### II. THE PNEUMOCOCCI AND NONHEMOLYTIC STREPTOCOCCI OF THE ADENOIDS AND TONSILS

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In the study of the incidence of the pneumococcus in the mouths of healthy persons it has been found that this organism is common in the saliva and throat. Park and Williams<sup>1</sup> identified typical pneumococci in about 50%; Longcope and Fox<sup>2</sup> and Buerger<sup>3</sup> gave similar percentages. Dochez and Avery<sup>4</sup> found pneumococci in 58.4% and Stillman<sup>5</sup> in about 45%, the great majority of which were types 4 and 3. The investigations were made on the saliva and less often on swabs of the throat and pharynx of normal people. In a study of the hemolytic streptococci of the adenoids and tonsils it was noted that streptococci were present in larger numbers in the crypts and folds than in the swab cultures of the same structures before their removal. To determine more accurately the true incidence of the pneumococci in the oro- and naso-pharynx cultures were made of the extirpated adenoids and tonsils.

The same material from which the hemolytic streptococci were isolated was cultivated on blood-agar plates, both streaked and poured, and studied for the pneumococci and nonhemolytic streptococci. Nasopharyngeal swabs, the excised adenoids and tonsils from 21 persons were first cultivated; the extirpated adenoids of 82 persons were also investigated as regards their flora, on the surface as well as the depths, between the vegetations. The patients were children from 5 to 16 years of age, who presented tonsils and adenoids of varying degrees of hypertrophy with no other marked gross changes.

Received for publication Feb. 16, 1921.

<sup>1</sup> Jour. Exper. Med., 1905, 7, p. 403.

<sup>2</sup> Ibid., p. 430.

<sup>3</sup> Ibid., p. 497.

<sup>4</sup> Ibid., 1915, 22, p. 105.

<sup>5</sup> Ibid., 1916, 24, p. 651.

This material approaches the normal as nearly as possible. The specimens were obtained during the months of April, May and June, 1920.

Green-producing cocci of three types were encountered; small round, gray, discrete, biconvex, glistening colonies surrounded by a grayish-green zone corresponding to the viridans type of streptococcus; flatter, often checker-like or umbilicated colonies revealing usually lancet-shaped diplococci surrounded by a similar zone of methemoglobin, corresponding to the pneumococcus group; lastly, somewhat larger green moist colonies, often mucoid, showing a marked tendency to become confluent, some corresponding in their appearance to the pneumococcus mucosus, others to the so-called "Mathers" type of streptococcus. Confirmatory studies by means of bile solubility and inulin fermentation were carried out on the isolated pure cultures. Most of the flat checker-like colonies proved to be bile-soluble and to ferment inulin, while the convex type were bile-insoluble and noninulin fermenting; but some exceptions were noted in both. The bile soluble, inulin fermenting strains were classified as pneumococci, and the insoluble, nonfermenting strains as Streptococci viridans.

THE INCIDENCE OF STREPTOCOCCUS VIRIDANS AND PNEUMOCOCCUS IN CULTURES OF NASOPHARYNGEAL SWABS, ADENOIDS AND TONSILS

	Number of Persons	Percentage Positive	
		Pneumococcus	Streptococcus viridans
Series 1. Swabs.....	21	71.4	90.5
Adenoids.....	21	71.4	90.5
Tonsils.....	21	66.6	81.0
Series 2. Adenoids.....	82	62.3	88.0
Total number adenoids examined	103	65.0	89.0

In the first series of 21, pneumococci occurred in 16 of the swab cultures, in 16 of the adenoids cultures and in 14 of the cultures of the tonsillar crypts. Streptococcus viridans was found in 19 of both swab and adenoid cultures, and in 17 of the tonsil cultures. The Mathers type of streptococcus was encountered once in all. The results are indicated in the table. In the second series of 82 adenoids pneumococci were encountered in 51, streptococcus viridans in 72. It is interesting to observe that in the nasopharyngeal swabs the pneumococci seldom were predominant, whereas in the adenoids from the same persons they were quite numerous in 4 instances and in almost pure culture in 2. Occasionally they were also numerous in the tonsillar crypts.

The crypt-like depressions of the adenoids seemed to harbor larger numbers. In two instances in which the pneumococcus and streptococcus viridans were absent in the tonsils although present in the adenoids, hemolytic streptococci were recovered in pure culture from the crypts. No great variations were noted in the numbers of streptococcus viridans excepting that in the crypts of the tonsils they were often fewer than in the nasopharynx.

In 12 instances typical streptococcus colonies revealing gram-positive cocci in chains were encountered, causing neither hemolysis nor methemoglobin formation.

Forty strains of *Streptococcus viridans* were inoculated in litmus milk and carbohydrate broth. All acidified and coagulated milk in from 7 to 10 days. The sugar medium contained 1% carbohydrate in infusion broth with Andrade's indicator. Lactose was fermented by all, mannite by 3, salicin by 14. According to Holman's classification, 6% are *Strep. fecalis*, 30% *Strep. motis* and 64% *Strep. salavarius*.

The moist green-forming colonies were somewhat variable in their appearance. Altogether they were encountered in the adenoids of 26 persons. Nine were distinctly moist mucoid colonies like the *Pneumococcus mucosus*. The other 17, though quite moist, showed a tendency toward flattening and conformed to the description of the so-called Mathers streptococcus. Of the former 9 strains, 6 were bile soluble, 3 insoluble. The 6 strains conform to the true *Pneumococcus mucosus*; the other 3 might possibly be termed *Streptococcus mucosus*. Of the 17 Mathers type, 9 were bile soluble, 8 insoluble, 6 fermented inulin and 11 did not. Morphologically, most of these organisms revealed gram-positive capsulated diplococci often lanceolate shaped; the Mathers coccus frequently appeared in chains of variable length.

Agglutination with specific types 1, 2, and 3 serums from the Rockefeller Institute and the New York State Board of Health was carried out on the bile-soluble organisms. Of the total 67 strains, none was agglutinated by type 1, 2 by type 2 and 10 by type 3. Two strains clumped by type 3 serum were not typical mucoid like the *Pneumococcus mucosus*. Two others were of the Mathers type.

Of the 14 strains from the tonsil, 2 were type 3, 12 were type 4.

#### SUMMARY

To ascertain more accurately the incidence of pneumococci in the throat and nasopharynx cultures were made from the extirpated adenoids and tonsils.

In a series of 103 adenoids, pneumococcus occurred in 65%, 2% of which were type 2, 13% type 3 and 85% type 4. In the nasopharyngeal swabs of 21 persons the pneumococcus was recovered in 71.4% ; from the tonsils of the same persons in 66.6%, and the adenoids in 71.4%. It was observed that in the depths of the folds and the crypt-like depressions of the nasopharyngeal vegetations and from the tonsillar crypts the pneumococci were decidedly more numerous than in the swabs. In 4 instances the pneumococci occurred practically in pure culture from the adenoids.

*Streptococcus viridans* was found in 89% of the adenoids and 81% of the tonsils. *Streptococcus mucosus* was encountered in 3% of the adenoids, and indifferent streptococci in 12%. The Mathers coccus was noted in 17% of the adenoids, once in pure culture.

The adenoids and tonsils are foci in which pneumococci and nonhemolytic streptococci commonly flourish.

# BACTERIOLOGIC STUDIES OF THE UPPER RESPIRATORY PASSAGES

## III. THE INFLUENZA BACILLI (PFEIFFER) OF THE ADENOIDS AND TONSILS

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Influenza bacilli have frequently been cultivated from the throat and sputum both from normal persons and from patients affected with various respiratory diseases. The incidence in normal persons varies somewhat according to different observers. Thus, in 1907 Davis<sup>1</sup> noted the organisms in 2 of 20 normal throats (10%). More recently Pritchett and Stillman<sup>2</sup> found the bacilli in 42% of 177 normal persons, and in school girls and boys of an orphan asylum, Winchell and Stillman<sup>3</sup> noted them in 25% to 39%. Opie and associates<sup>4</sup> give the incidence of Pfeiffer bacilli in from 24% to 35.1% of healthy soldiers. Lord<sup>5</sup> obtained the organism in as high as 76% of the swab cultures of 34 normal young men, while Jordon<sup>6</sup> recovered them in 40% of normal throats. From well children most of whom were under 2 years of age Wollstein and Spence<sup>7</sup> state that the bacilli occurred in 10% of 266. All of these figures were based on studies of the saliva or swab cultures of the pharynx and occasionally of the nasopharynx.

In previous studies of the extirpated tonsils and adenoids it was indicated that occasionally direct cultures of these structures would reveal hemolytic streptococci which were apparently too few on the surface to be recovered in the swab cultures of the same persons. To determine more accurately the frequency of the influenza bacillus in the oro- and naso-pharynx the excised tonsils and adenoids were cultivated with special reference to this organism. The material consisted of adenoids and tonsils consisting of lymphoid tissue revealing varying

Received for publication Feb. 16, 1921.

<sup>1</sup> Jour. Am. Med. Assn., 1907, 48, p. 1563.

<sup>2</sup> Jour. Exper. Med., 1919, 29, p. 259.

<sup>3</sup> Ibid., 1919, 30, p. 497.

<sup>4</sup> Jour. Am. Med. Assn., 1919, 72, p. 108.

<sup>5</sup> Ibid., p. 188.

<sup>6</sup> Ibid., p. 1542.

<sup>7</sup> Am. Jour. Dis. Child., 1920, 19, p. 459.

degrees of hyperplasia but no other marked pathologic change. The patients were children who presented no acute evidences of sore throat or any elevation of normal temperature. The age varied from 5 to 16 years, and the patients had had their tonsils and adenoids removed during the months of April, May, June and July, 1920. In 25 instances naso-pharyngeal swabs were made by means of the West tube immediately before the operation. Altogether cultures were made from the adenoids and tonsils from each of 115 persons.

The material was collected in sterile gauze and cultures were made within 4 hours after removal. Cultures were made from the surface of the adenoids and from depths between the folds and cryptlike slits which frequently occur in their structure. The tonsils were incised with a sterile knife transversely to the long axis of the crypts and cultures made from the bottom of the crypts. Ten per cent. blood agar for pouring into Petri dishes were inoculated, as well as the surface of blood-agar plates. Additional cultures were made on the so-called chocolate medium consisting of infusion agar to which defibrinated human blood was added in 5% proportion and the whole heated at 90 C. for 5 minutes.

On the chocolate plates the influenza bacilli occurred in the cultures of the adenoids in 47 instances or 40.9% and of the tonsils in 62 or 53.9%. In the nasopharyngeal swabs they were found in 10 or 40%. In each instance in which the bacilli appeared in the adenoids they were also recovered from the tonsils. From the depths of the adenoid folds the organism was isolated in one instance but did not grow out on the cultures of the surface or the nasopharyngeal swab. The brown plates frequently revealed these organisms in large numbers growing as gray, often flat, colonies of variable size, not infrequently being the predominating bacterium on this special medium. On the streaked and poured blood-agar plates the bacilli grew as small translucent dewdrop-like colonies occasionally showing strikingly the tendency to grow in clusters immediately around other colonies observed most readily in the zones of hemolysis of the *Streptococcus hemolyticus*. This symbiotic tendency was seen only when the influenza bacilli occurred in relatively large numbers, for in many instances a few transparent colonies revealing gram-negative pleomorphic bacilli were observed without this tendency. In several instances the bacilli were apparently absent on the fresh blood agar, while they were present in few or even moderate numbers on the heated blood medium.

The bacilli appeared as gram-negative small rods, some strains showing a uniform size, and a few exhibited an extreme tendency toward larger and thread-like forms, while several displayed coccoid forms. Pure cultures, isolated on chocolate-agar slants, revealed apparently more marked pleomorphism. Each strain was inoculated on a plain infusion-agar slant, and in no instance did growth occur. They were further studied by inoculating the entire surface of a fresh unheated blood-agar slant on which another organism like the staphylococcus albus was subsequently streaked linearly. In each instance the influenza bacilli grew as small dew-drop colonies which were largest and most numerous immediately adjacent to the linear streak of the foreign organism demonstrating the property of symbiosis so characteristic of the Pfeiffer bacillus.

THE INCIDENCE OF INFLUENZA BACILLUS (PFEIFFER) IN THE CULTURES OF NASOPHARYNGEAL SWABS, ADENOIDS AND TONSILS

	Number of Persons	Percentage Positive
Series I. Swabs.....	25	40.0
Adenoids.....	25	44.4
Tonsils.....	25	48.0
Series II. Adenoids.....	90	38.8
Tonsils.....	90	55.5
Total number of adenoids from which cultures were made.....	115	40.9
Total number of tonsils from which cultures were made.....	115	53.9

It is interesting to observe that the influenza bacilli occurred more often in the crypts of the tonsils and the crypt-like structures of the adenoids than in the nasopharyngeal swabs or cultures from the surface or folds of the adenoids. The organisms were also inclined to be decidedly more numerous in the depths of these structures than on the surface. In these respects the Pfeiffer bacillus resembles the streptococcus hemolyticus, illustrating the rôle played by the lymphoid structures of the oro- and naso-pharynx in furnishing foci in which dangerous bacteria flourish.

#### SUMMARY

Gram-negative, pleomorphic, hemoglobinophilic bacilli, showing a preference for heated blood agar and revealing the characteristic prop-

erty of symbiosis, were isolated and identified in 40.9% of extirpated adenoids and in 53.9% of the excised tonsils from 115 persons. In the nasopharynx they were present in 40% of 25 persons and in fewer numbers.

The tonsils and adenoids therefore are foci in which influenza bacilli (Pfeiffer) commonly flourish.



## BACTERIOLOGIC STUDIES OF THE UPPER RESPIRATORY PASSAGES

### IV. THE INCIDENCE OF PNEUMOCOCCI, HEMOLYTIC STREPTOCOCCI AND INFLUENZA BACILLI (PFEIFFER) IN THE NASOPHARYNX OF TONSILLECTOMIZED AND NONTONSILLECTOMIZED CHILDREN

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It was noted previously by Pilot and Davis<sup>1</sup> that the incidence of hemolytic streptococci was less in the oropharynx of tonsillectomized than in the nontonsillectomized, the organisms occurring in 15.8% in few numbers in the former group as compared with 58% in larger numbers in the latter group. Nichols and Bryan<sup>2</sup> reported the disappearance of these organisms from the throat in 27 of 31 patients 11 days after extirpation of diseased tonsils. Simmons and Taylor<sup>3</sup> noted the streptococci in fewer numbers in a somewhat larger percentage (23%) after tonsillectomy. Tongs<sup>4</sup> found these streptococci in 5% of tonsillectomized persons as compared with 60% of the cultures of the surface of the tonsils of the nontonsillectomized. Van Dyke<sup>5</sup> obtained positive cultures in 16.4% of tonsillectomized persons, mostly adults. In all of these investigations cultures were made of the pharynx or the region of the tonsils, and studied with special reference to the incidence and numbers of hemolytic streptococci. In all it is quite evident that tonsillectomy reduces considerably the frequency of *Streptococcus hemolyticus*. As no nasopharyngeal cultures were made, a study was undertaken of the flora of the nasopharynx with reference to the pneumococcus and the influenza bacillus, as well as the hemolytic streptococcus in children whose tonsils and adenoids had been removed, and a comparison made with a similar group in which tonsils and adenoids were present. The investigation was carried out from September to December, 1920.

Received for publication March 17, 1921.

<sup>1</sup> J. Infect. Dis., 1919, 24, p. 386.

<sup>2</sup> Jour. Am. Med. Assn., 1918, 71, p. 1872.

<sup>3</sup> Ibid., 1919, 72 p. 1885.

<sup>4</sup> Ibid., 1919, 73, p. 1050.

<sup>5</sup> Ibid., 1920, 74, p. 448.

The children studied were normal girls and boys from 5 to 15 years of age of the Marks Nathan Orphan Home. In some the tonsils and adenoids had been removed from 2 months to 5 years previously. All the children had normal temperature and no subjective or objective evidences of acute inflammation of the throat or respiratory passages. The nasopharynx was swabbed with a curved wire swab which was spread on 10% human blood-agar and 5% heated blood-agar plates, and then placed in infusion broth and incubated for 24 hours. The infusion broth cultures were then inoculated into melted blood agar and poured plates made. The blood-agar plates were examined particularly for the pneumococcus, the heated blood-agar plates for the influenza bacillus and the poured plates for the hemolytic streptococcus (Table 1).

TABLE 1  
THE INCIDENCE OF PNEUMOCOCCI, HEMOLYTIC STREPTOCOCCI AND INFLUENZA BACILLI IN  
TONSILLECTOMIZED AND NONTONSILLECTOMIZED CHILDREN

Organisms	Number of Persons	Tonsillectomized Percentage Positive	Number of Persons	Nontonsillectomized Percentage Positive
Pneumococcus .....	49	32.5	68	32.3
Streptococcus hemolyticus .....	27	40.8	40	60
B. influenzae .....	29	26.5	35	37.1

*Pneumococcus*.—Small flat checkered colonies were isolated and inoculated into plain broth. They were then studied as to morphology, bile solubility, inulin fermentation and specific agglutination with types 1, 2 and 3 serums. In 49 children without tonsils and adenoids pneumococci were identified in 15 (32.5%). Ten of these strains were type 4, 2 type 3, 1 type 2a and 2 type 1. In one instance a green streptococcus insoluble in bile, but which fermented inulin, was found. In the 68 with tonsils and adenoids the pneumococcus occurred in 21 (32.3%), of which 20 were type 4 and one type 2a. In two instances a green streptococcus bile-insoluble but fermenting inulin was encountered. In 8 children whose throats revealed remnants of tonsils the pneumococcus was present in 3; two of the strains were of type 4, the other type 3. It was noted throughout that while the percentage of pneumococci did not differ in the 2 groups, the number of colonies of pneumococci when present was fewer in the cultures from the tonsillectomized than those from the nontonsillectomized.

*Streptococcus hemolyticus*.—Small gray colonies with zones of complete hemolysis from 1 to 4 mm. wide corresponding to the beta type of the streptococcus hemolyticus were isolated. These organisms fer-

mented lactose and salicin, but did not ferment inulin or mannite. They were pathogenic for rabbits, one blood-agar slant usually causing arthritis and often death in a young rabbit. They resembled in their properties the hemolytic streptococci of the crypts of the tonsils and adenoids. In 27 children whose tonsils and adenoids were absent the beta type of streptococcus was found in 11 (40.8%); the alpha type occurred in one instance. In 40 children with tonsils the beta type was present in 24 (60%) and in 2 the alpha was encountered. Of the 8 with remnants beta type was present in 3 and the alpha in 2. It is interesting to note that in the cases with tonsils the hemolytic streptococci were often present in moderate numbers only occasionally exceeding 10% of the total colonies on the plate. In the cultures from the children without tonsils the number of colonies was decidedly less, in none exceeding 10% of the total number and in 3 instances only a single colony was present.

*B. Influenzae*.—On the heated blood-agar plates typical gray, often flat, colonies were studied and subcultivated. The organisms were small gram-negative bacilli often pleomorphic, particularly on the subcultures. Transfers were made on infusion agar where no growth occurred when the organisms were true influenza bacilli. They were further subcultivated on unheated blood-agar slants and another organism (staphylococcus) streaked linearly on the same slant. The influenza colonies showed the property of symbiosis growing in larger size and numbers about the foreign organism. Of 29 persons whose tonsils were absent, 8 (26.5%) gave positive cultures for *B. influenzae*. Of 35 whose tonsils were present, 13 (37.1%) were positive. In the 8 with remnants, 4 gave positive results. Here also it is noteworthy that the number of colonies were more numerous in the cultures of the nontonsillectomized group.

#### SUMMARY

Pneumococci, hemolytic streptococci and *B. influenzae* were often found in the nasopharynx of normal children.

The incidence and numbers of hemolytic streptococci and influenza bacilli in the nasopharynx is decidedly less in the children whose adenoids and tonsils had been removed. In case of the pneumococcus the numbers are less in the same children than in those whose tonsils were present.

The removal of tonsils and adenoids reduces the number of certain bacteria in the oro-pharynx and naso-pharynx, but does not cause their disappearance.

## BACTERIOLOGIC STUDIES OF THE UPPER RESPIRATORY PASSAGES

### V. THE DIPHTHERIA BACILLI AND DIPHTHEROIDS OF THE ADENOIDS AND TONSILS

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The tonsils are the foci that usually harbor diphtheria bacilli in carriers, and their removal frequently terminates the carrier state.

Pegler<sup>1</sup> was perhaps the first to observe this effect, which has been described also by Friedberg<sup>2</sup> who reported 6 carriers that gave negative cultures for *B. diphtheriae* after extirpation of the tonsils and adenoids. Ruh, Miller and Perkins<sup>3</sup> obtained similar results in 19 cases and Rabinoff<sup>4</sup> had the same experience in 10 instances. In his study of diphtheria carriers Weaver<sup>5</sup> reports that cultures became negative in 40 persistent carriers within 18 days after the removal of the tonsils and adenoids. Keefer, Friedberg and Aronson<sup>6</sup> state that in 77.2% of the carriers they studied the organisms were in the tonsils, often in pure culture, and that 91.3% became negative within 2 weeks after tonsillectomy.

The diphtheria bacilli apparently are in the crypts of the tonsils as well as on the surface. Ruh, Miller and Perkins<sup>3</sup> call attention to 5 carriers who gave positive cultures of the crypts while the surface cultures were negative. Dwyer and Gignoux<sup>7</sup> obtained 5 positive reactions from direct cultures of the crypts of 72 persons. Brown<sup>8</sup> demonstrated in microscopic sections gram-positive bacilli morphologically like the diphtheria bacillus in the crypts and in the tissues beneath the thin epithelium of the tonsils of 7 carriers. Ballantyne and Cornell<sup>9</sup> found diphtheria bacilli in the tonsillar crypts of 6 carriers and once in the adenoids. Hartley and Martin<sup>10</sup> found the bacilli in sections of the tonsils, in the crypts but not in the tissues.

In all of these observations it is noteworthy that cultures were  
\* taken from the tonsils only occasionally, from the adenoids practically never, and apparently none of the bacilli identified was tested for

Received for publication April 7, 1921.

<sup>1</sup> Brit. Med. Jour., 1905, 2, p. 621.

<sup>2</sup> Jour. Am. Med. Assn., 1916, 66, p. 810.

<sup>3</sup> Ibid., p. 941.

<sup>4</sup> Ibid., 67, p. 1722.

<sup>5</sup> Ibid., 1921, 76, p. 831.

<sup>6</sup> Ibid., 1918, 71, p. 1206.

<sup>7</sup> Laryngoscope, 1910, 20, p. 1042.

<sup>8</sup> Jour. Infect. Dis., 1916, 19, p. 565.

<sup>9</sup> Brit. Med. Jour., 1917, 2, p. 686.

<sup>10</sup> Quoted by Weaver.<sup>5</sup>

virulence. To determine the frequency in the naso- and oro-pharynx of virulent and avirulent forms of diphtheria bacilli and of related organisms, a bacteriologic study with special reference to these points was made of the extirpated adenoids and tonsils of the same patients.

The material was obtained from 100 children who entered the Cook County Hospital to have their tonsils and adenoids removed chiefly because of hyperplasia. They were afebrile and had no evidences of acute inflammation of the throat or respiratory passages. They varied from 5 to 15 years of age, and were boys and girls living in scattered parts of Chicago. A reliable history of an attack of diphtheria was not to be obtained in any case. The adenoids and tonsils were removed during the months of October and November, 1920.

The material was selected and cultures were taken only from adenoids which were not macerated. Cultures were obtained from the tonsils of the same persons. Altogether 100 adenoids and 100 pairs of tonsils were studied. Cultures were made in from 1 to 3 hours after the operation.

The adenoids revealed no marked pathologic change other than hyperplasia. There were no gross evidences of necrosis or of a purulent exudate. They consisted of lymphoid tissue from 0.5 to 1.5 cm. in all dimensions, presenting 3 to 6 folds and often cryptlike depressions not unlike the crypts of the tonsils. The tonsils, like the adenoids, often showed marked hyperplasia, some however, being of normal size without any gross change. Occasionally fatty debris and cholesterol crystals, rarely pus, were observed in the crypts. In none was necrosis seen.

Cultures were taken from the surface of the epithelial lining of the adenoids and the crypts of the tonsils. From the adenoids cultures were obtained by passing a wire loop over the surface, and then after carefully separating the folds or orifices of the depressions another culture was taken of the sides and bottoms of these structures. The tonsils were incised transversely and cultures made with a loop from the bottoms of crypts. Slants of Loeffler serum medium with a broad surface were used and examinations made at the end of 18 to 24 hours.

The diphtheria bacilli and diphtheria-like organisms grew as small gray opaque colonies and were identified with the methylene blue and gram stains. Bacilli with typical polar granules corresponding to the C and D and occasionally A types of Wesbrook were indicated as diphtheria bacilli. In several instances the barred types C' and D' were encountered, but granular types were also present. The remaining

bacilli, all gram-positive, occurring often in palisades, were termed diphtheroids. Pure cultures were obtained from subcultures on blood-agar plates.

Diphtheria bacilli were recovered in 12 instances, both from the adenoids and tonsils (table 1). In one case the bacilli were found on the surface of the adenoids and not in the depths. In 5 instances the bacilli occurred in one tonsil and not in the other. In all instances when the bacilli were present they appeared in the adenoids as well as in one or both tonsils. The organisms on Loeffler's medium appeared frequently in pure culture or in predominating numbers (table 2). In the crypts of the tonsils they generally occurred in larger numbers than in the adenoids. It is of interest to note that two strains which proved virulent occurred in practically pure culture in both the adenoids and tonsils.

TABLE 1

THE INCIDENCE OF THE *B. DIPHThERIAE* AND DIPHThEROIDS IN THE EXCISED ADENOIDS AND TONSILS OF 100 CHILDREN

	<i>B. diphtheriae</i> , Percentage Positive	Diphtheroids, Percentage Positive
Surface of the adenoids.....	12	25
Depths of the adenoids.....	11	24
Surface and depths of the adenoids.....	12	30
Right tonsil.....	12	14
Left tonsil.....	7	11
One or both tonsils.....	12	17

TABLE 2

RELATIVE NUMBERS OF *B. DIPHThERIAE* IN THE ADENOIDS AND TONSILS OF 100 CHILDREN

	Surface of Adenoids, Percentage	Depths of Adenoids, Percentage	Right Tonsil, Percentage	Left Tonsil, Percentage
Pure culture.....	3	2	5	2
Predominating numbers.....	3	2	2	2
Moderate numbers.....	1	3	3	2
Few numbers.....	5	4	2	1
None.....	88	89	88	93

On blood agar 4 strains caused hemolysis; only one proved virulent. In 1% carbohydrate broth with Andrade indicator all 12 strains produced acid in dextrose and dextrin, 10 in maltose and lactose and none in saccharose and mannite.

Two strains were repeatedly virulent in doses of 1, 2 and 3 c c of 48-hour cultures of infusion broth for 300 to 400 gm. guinea-pigs, causing death within 24 hours with typical local edema and hemorrhage,

fluid in pleural cavities and hemorrhagic adrenals. The antitoxin controls inoculated with 250 units survived in all instances. A third strain showed low virulence, killing in doses of 3 and 5 c c serum broth, while the antitoxin controls survived. Three other strains in large doses (5 c c) were pathogenic, producing local abscesses, hemorrhages in mucous membranes and muscles with congestion of the kidneys and adrenals, less marked, however, than in the lesions of the more virulent strains. Subsequent cultures were totally avirulent. The remaining 6 strains were avirulent. The 3 strains of low pathogenicity correspond closely to the bacilli described by Hamilton<sup>11</sup> in otitis media complicating scarlatina.

In this connection it is worthy to compare the incidence of virulent and avirulent diphtheria bacilli in swab cultures of the throats of normal persons determined by several investigators with the incidence in the adenoids and tonsils. It should be remembered that a surface swab culture has been repeatedly shown not to be a true index of the flora in the crypts of the tonsils in which the organisms are present in larger numbers free from contamination of the bacteria of the saliva and sputum. It is also important to note that most of the statistics available are based on a single swab culture and that the percentage undoubtedly would be higher if cultures of the nasopharynx, each tonsillar surface and the pharynx were taken separately. The report of the Massachusetts Association of Boards of Health<sup>12</sup> indicates a percentage of 1 to 2% of the urban population and 5 to 8% in institutions, and of the strains tried 17% were found virulent. Pennington<sup>13</sup> found that 9.3% of 375 school children carried the bacilli, of which 14% were virulent and 30% of attenuated virulence. Von Sholly<sup>14</sup> obtained in the cultures of 1,000 normal throats virulent bacilli in 1.8% and nonvirulent in 3.8%. Goldberger, Williams and Hachtel<sup>15</sup> report *B. diphtheriae* in 0.928% of 4,093 healthy persons, of which of 19 strains tested 10.5% were virulent. Guthrie, Gelien and Moss<sup>16</sup> encountered the organisms in 3.55% of 2,507 children and adults, of which 18.18% of 33 strains were virulent. In general, the bacilli recovered from normal persons not convalescent, or contacts from 10.5 to 18.18%, were virulent comparing closely to the strains of the adenoids and tonsils, of which 16.66% were virulent.

<sup>11</sup> Jour. Infect. Dis., 1907, 4, p. 316.

<sup>12</sup> Jour. Mass. Assn. of Boards of Health, 1902, p. 1202.

<sup>13</sup> Jour. Infect. Dis., 1907, 4, p. 36.

<sup>14</sup> Ibid., 1907, 4, p. 337.

<sup>15</sup> Bull. 101, Hyg. Lab., 1915, p. 29.

<sup>16</sup> Bull. Johns Hopkins Hosp., 1920, 357, p. 388.

The diphtheroid bacilli appeared in the smears as nongranular, shorter and stouter than the Klebs-Loeffler organism. In the adenoids they occurred in 30% and in the tonsils in 17% (table 1); 9 of the 30 and 4 of the 17 resembled the D2 type of Westbrook or the Hoffman bacillus. In the adenoids the diphtheroids were usually encountered in larger numbers on the surface than in the depths. In the crypts of the tonsils these organisms were decidedly less common and numerous (table 3). In the 13 instances in which the diphtheroids were absent in the crypts, although present in the adenoids, the predominating growth of the crypt cultures were coccus forms chiefly streptococci. The greater incidence of the diphtheroid group in the nasopharynx than in the tonsils is of interest for it has often been observed that these organisms are more common in the nose and nasal passages than in the oropharynx (Gorham<sup>17</sup>). Twenty strains isolated in pure culture did not ferment dextrose, lactose, maltose, saccharose or dextrin resembling the strains from the tonsils studied by Eberson.<sup>18</sup> None of the strains were hemolytic.

TABLE 3  
RELATIVE NUMBERS OF DIPHTHEROIDS IN THE ADENOIDS AND TONSILS OF 100 CHILDREN

	Surface of Adenoids, Percentage	Depths of Adenoids, Percentage	Right Tonsil, Percentage	Left Tonsil, Percentage
Pure culture.....	3	3	0	0
Predominating numbers.....	9	2	0	0
Few or moderate numbers.....	20	19	14	11
None.....	75	76	86	89

#### SUMMARY

Cultures made of the excised adenoids of 100 children revealed *B. diphtheriae* in 12.

The crypts of the extirpated faucial tonsils of the same persons harbored the bacilli in 12. When present in the tonsils the bacilli also occurred in the adenoids of the same person.

In the tonsillar crypts the diphtheria bacilli were usually more numerous than in the adenoids.

Two of the 12 strains were virulent; one showed attenuated virulence; three were pathogenic in large doses of the first culture while subsequent cultures were without virulence; the remainder were totally avirulent.

Diphtheroids occurred in 30 of the adenoids and in 17 of the tonsils; when present in both they were decidedly more numerous in the nasopharyngeal vegetations than in the tonsillar crypts.

<sup>17</sup> J. Med. Res., 1901, 6, p. 201.

<sup>18</sup> Jour. Infect. Dis., 1918, 23, p. 14.



# TREHALOSE FERMENTATION IN THE DIFFERENTIATION OF THE PARATYPHOID-ENTERITIDIS GROUP

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The investigations of Bainbridge,<sup>1</sup> Savage,<sup>2</sup> Jordan,<sup>3</sup> Krumwiede and his associates<sup>4</sup> and others have done much to clarify our knowledge of the confusing paratyphoid-enteritidis group, and as a result of this work a number of well-defined types are now recognized. A summary of this group with suggested changes in nomenclature is given by Winslow, Kligler, and Rothberg.<sup>5</sup> Recently, Ten Broeck<sup>6</sup> has called attention to a group of paratyphoids of animal origin, which he found to be culturally identical to *B. paratyphosus* B, or *B. schottmulleri*, but which may be differentiated by agglutinin absorption tests. Many of the paratyphoid B bacilli isolated from food poisoning cases quite possibly belong to the same group and Ten Broeck suggests that "if it is found upon further study that these organisms are the same as the *Aertrycke* bacillus, isolated by DeNobele, the name of *Bacillus aertryckei* would be appropriate." An apparently similar group was observed by Krumwiede, Valentine and Kohn<sup>7</sup> who by absorption tests separated from *B. schottmulleri* a number of strains of rodent origin.

Thus, while the various members of the paratyphoid-enteritidis group can be separated one from the other by serologic methods, the distinctions based on cultural or biochemical features are in some instances slight or altogether lacking. For this reason it is believed that any further means of differentiation may be of distinct practical value. While engaged in identifying an organism of this group asso-

Received for publication Feb. 16, 1921.

1. Jour. Pathol. and Bact., 1908-09, 13, p. 443. Bainbridge, F. A., and O'Brien, R. A.: Jour. Hygiene, 1911, 11, p. 68.

2. Jour Hygiene, 1912, 12, p. 1.

3. Jour. Infect. Dis., 1917, 20, p. 457; 1918, 22, p. 511; 1920, 26, p. 427. Jordan, E. O., and Victorson, R.: Idem., 1917, 21, p. 554.

4. Krumwiede, C.; Pratt, J. S., and Kohn, L. A.: Jour. Med. Res., 1916-17, 35, 55, p. 357. Krumwiede, C.; Kohn, L. A., and Valentine, E.: Idem., 1918, 38, p. 89.

5. Jour. Bacteriol., 1919, 4, p. 429.

6. Jour. Exper. Med., 1918, 28, p. 759; 1920, 32, p. 19.

7. Jour. Med. Res., 1919, 39, p. 449.

ciated with a case of human food poisoning, it was observed that certain types could be differentiated on the basis of their abilities to ferment trehalose. It is my purpose to present these results.

Trehalose is a disaccharide sugar consisting of 2 molecules of glucose attached in such a way that both aldehyde groups have disappeared.<sup>8</sup> It does not reduce Fehling's solution. Two different samples of trehalose were available for this study. The one was procured from a commercial firm, the other was prepared in the Carbohydrate Laboratory of the Bureau of Chemistry. Results obtained by the use of either sample were checked by similar tests with the other. The 2 samples gave identical results.

The strains of the several types of the paratyphoid-enteritidis group employed in this investigation were for the most part stock cultures received from various laboratories. The writer is especially indebted to Dr. K. F. Meyer and to Dr. Carl Ten Broeck for a number of the para B type cultures of animal origin. The term "animal para B cultures" is used in this paper to designate the group of paratyphoid B cultures of animal origin which may be differentiated from *B. schottmulleri* by agglutinin absorption tests.<sup>6, 7</sup> This expression is used here for convenience only, and in the absence of definite knowledge that these cultures are similar to the *B. aertryckei* of de Nobele. It should be noted that cultures identical to *B. schottmulleri* have been reported as obtained from animal sources.<sup>9</sup> One of these, 135 "B," is included in the present investigation.

In table 1 is shown the deportment of the various members of the paratyphoid-enteritidis group toward trehalose when incorporated in the ordinary culture mediums. For comparison, results are also given for the test substances usually employed in the differentiation of these types. Here it is seen that *B. suipestifer*, in contrast to the other members of this group, is incapable of attacking trehalose. On continued incubation of the trehalose broth, an apparent reversion to an alkaline reaction was observed in many of the *B. schottmulleri* cultures. The brilliant red color of the Andrade indicator gradually faded and eventually disappeared. On the basis of what was probably a similar loss of color in old cultures, Krumwiede, Pratt and Kohn<sup>10</sup> devised their glucose

<sup>8</sup> Armstrong, E. Frankland: *The Simple Carbohydrates and the Glucosides*, Ed. 3, 1919, p. 101.

<sup>9</sup> Spray, R. S.: *Jour. Infect. Dis.*, 1920, 26, p. 340.

<sup>10</sup> *Jour. Med. Res.*, 1917, 35, p. 357.

serum water medium. This suggested to the writer the possibility of a similar means for differentiating the animal para B cultures from *B. schottmulleri*.

The first procedure tried for the separation of the two groups referred to in the foregoing was the substitution of trehalose for glucose in Krumwiede's glucose serum water medium.<sup>11</sup> After some experimentation it was found that a sharp distinction could be brought out between *B. schottmulleri* and the animal para B type, provided that certain conditions were fulfilled. The most desirable procedure thus far found has been to add the trehalose to the serum water in sufficient

TABLE 1  
ACTION OF MEMBERS OF PARATYPHOID-ENTERITIDIS GROUP TOWARD TREHALOSE

Types	Number of Strains	Fermentation of							
		Xylose		Arabinose		Dulcitol		Trehalose	
		+	-	+	-	+	-	+	-
<i>B. paratyphosus</i> (para A)	3	0	3	3	0	3 (slow)	0	3	0
<i>B. schottmulleri</i> (paratyphosus B)	7	7	0	7	0	7	0	7	0
Animal para B type	14	13 1 (3 days)	0	14	0	14	0	10 2 (48 hrs.) 2 (2 days)	0
<i>B. enteritidis</i>	6	6	0	6	0	6	0	6	0
<i>B. suispestifer</i>	8	8	0	0	8	1 (rapid) 2 (slow)	5	0	8

For these tests meat extract peptone broth,  $P_H$  7.0-7.2, to which was added 1% of Andrade indicator was used. The sugars and the alcohol, dulcitol, were sterilized separately in 10% solution in distilled water and added aseptically to the broth tubes in sufficient amounts to give a concentration of 0.5%. The tests were observed for 2 weeks in order to include any delayed fermentation. All fermentation results recorded as positive occurred within 24 hours unless otherwise designated.

quantities to give a concentration of 0.5% and to tube the resulting trehalose serum water medium in small quantities to insure a shallow layer of medium, preferably not more than 1.5 cm. in height. After 3 to 4 days' incubation at 37 C., the coagulum produced by the animal para B cultures presents a deep pink or red color, while that of the *B. schottmulleri* cultures is colorless or a light pink tint. This distinction is not noticeable after 24 hours, but is gradually brought out on further incubation. It has been the writer's experience that the longer the period

<sup>11</sup> For the convenience of the reader, the composition of this medium is repeated here: one part sterile horse serum and 4 parts sterile distilled water to which are added 1% Andrade indicator and sufficient amounts of a sterile glucose solution to give a final concentration of 0.1%. The medium may be prepared from sterile materials and tubed aseptically or prepared from nonsterile materials and given a 3-day intermittent heating in the Arnold sterilizer.

of incubation, up to one week, the greater is the contrast between the two types. After that time no additional changes have been observed. Most of the animal para B cultures have been found to be somewhat slower in coagulating the serum than are the *B. schottmulleri* strains. In the case of a few of the animal paratyphoid cultures which required 48 to 72 hours for a noticeable fermentation of trehalose (table 1), the typical appearance in the trehalose serum medium has been delayed for

TABLE 2  
SHOWING CORRELATION OF BIOCHEMICAL AND SEROLOGIC DIFFERENTIATION OF THE "ANIMAL PARA B" CULTURES AND *B. SCHOTTMULLERI*

	Agglutinin Absorption Tests				Fermentation of Trehalose: Serum Water, 1% Andrade Indicator, 0.5% Trehalose. 4 Days at 27 C.
	B. schottmulleri Serum "Rowland" Absorbed by		Calf Typhus 1 Serum Absorbed by		
	Nothing (Control)	Calf Typhus 1	Nothing (Control)	B. schott- mulleri (Rowland)	
B. schottmulleri					
Am. Mus. ....	6,400	1,600	800	200—	Faint pink
Cool's Army. ....	6,400	1,600	3,200	200—	White
Roanoke. ....	6,400	3,200	1,600	200—	Faint pink
Rowland. ....	6,400	1,600	1,600	200—	White
Jordan 210. ....	6,400	2,200	1,600	200—	Faint pink
F. ....	6,400	1,600	1,600	200—	Faint pink
Perdue, 135 B. ....	6,400	1,600	3,200	400	Faint pink
Animal para-B					
Calf-typhus 1. ....	3,200	200—	6,400	3,200	Red
Swine-typhus 1. ....	3,200	200—	6,400	3,200	Red
Mouse-typhus 1. ....	3,200	200	6,400	3,200	Red
Swine-typhoid 2-1. ....	3,200	200	6,400	1,600	Red
Swine-typhoid 2-2. ....	1,600	200	6,400	3,200	Deep pink
Pigeon 6. ....	3,200	400	6,400	1,600	Red#
Canary 12. ....	6,400	400	6,400	1,600	Deep pink
Psittacosis, Past.					
Inst. ....	3,200	200	6,400	800	Deep pink#
Guinea-pig 10. ....	3,200	200	6,400	3,200	Deep pink
Guinea-pig 479. ....	3,200	200	3,200	1,600	Red
Guinea-pig 480. ....	3,200	200	6,400	1,600	Red+
Rabbit 1371. ....	3,200	200	6,400	3,200	Deep pink
Rabbit 45. ....	3,200	200	3,200	1,600	Deep pink+
Anatum (Rettger)...	1,600	200	6,400	3,200	Red

Agglutination tests: Immune rabbit serums used. Figures represent the highest dilution at which definite clumping was observed. 200— = no clumping at 1:200. Dilutions lower than 1:200 were not made.

Trehalose serum water: Coagulation of serum in all tubes with the production of the color indicated.

#, + = slow fermenters (table 1); typical appearance delayed 3 days = (#) or 2 days = (+).

a corresponding interval. This deep pink or red color of the animal para B cultures is first apparent at the surface of the coagulum, so that if a deep column of medium is used the typical coloration may appear only in the upper part of the coagulum with the result that much of the sharpness of the distinction is lost. The exact percentage of trehalose necessary to bring about this reaction has not been determined. When 0.1% trehalose was used, all the cultures remained color-

less or a light pink, and the distinction between the 2 types was then not manifest. One series of tests in which a concentration of 0.3% was employed gave a fairly well-defined distinction. In the greater part of this work 0.5% has been used.

Several repeated trials of this method with different lots of horse serum have given consistent results, and in every case the distinction based on this reaction in the trehalose serum water medium has correlated with the grouping by agglutinin absorption (table 2). Further, it is worthy of note that in this medium all the strains of *B. enteritidis* presented an appearance identical to that of the "animal para B" cultures and in contrast to *B. schottmulleri*. *B. suispestifer*, which is unable to attack trehalose, produced no change in the medium. The few *B. paratyphosus* cultures used in this investigation have coagulated the medium with the immediate production of either a deep pink or red color, i. e., no reduction of the Andrade indicator.

The hydrogen-ion concentration attained in a medium consisting of 0.5% peptone, 0.5% dipotassium hydrogen phosphate, and 0.5% trehalose has been found to be of some value in separating the animal para B cultures from *B. schottmulleri*, although it has not given as reliable and consistent a demarcation as has the trehalose serum water. Different peptones, Witte and Difco, have yielded different results. Also, the 4 cultures previously noted as slow fermenters of trehalose are exceptions and do not permit a uniform separation of these 2 types. When Witte peptone was used in the medium referred to in the foregoing the majority of the animal para B cultures attained a hydrogen-ion concentration of 5.0 to 5.2 after 48 hours at 37 C., while that of the *B. schottmulleri* strains varied from 5.6 to 5.8. On the addition of 4 or 5 drops of a 0.02% solution of methyl red to tubes containing 4-5 c c of a 48-hour culture, the animal para B cultures, with the exception of the slow fermenters, exhibited a pink color, in contrast to the light orange of the *B. schottmulleri* cultures. When Witte peptone was replaced by Difco, different results were secured with different lots of the latter brand of peptone. With one lot the results were quite similar to those obtained with Witte peptone. In others (a different bottle of the Difco peptone) four days' incubation were required before a distinct separation could be brought out between the two types. In this case, the *B. schottmulleri* cultures were yellow in contrast to the pink of the majority of the animal para B types. All of the strains of *B. enteritidis* throughout the numerous trials of this medium have attained

a hydrogen-ion concentration similar to that of the "animal para B type" and in contrast to *B. schottmulleri*. Because of the several discrepancies mentioned, this method is not recommended as one for classification of these types, although the results, especially when considered with those obtained by the use of the trehalose serum water medium, are suggestive.

#### SUMMARY

In a study of the availability of trehalose for the various members of the paratyphoid-enteritidis group, it was found that *B. suipestifer* is unable to attack this disaccharide, whereas *B. paratyphosus*, *B. schottmulleri*, the animal para B sub-group, and *B. enteritidis* ferment trehalose with the production of acid and gas.

Furthermore, it is possible to differentiate by cultural methods the *B. schottmulleri* strains from the closely allied animal para B group, hitherto separable only by serologic means. This has been accomplished by employing small amounts of a serum water medium containing 0.5% trehalose and 1% Andrade indicator. In this medium the animal para B strains produce a red coagulum after 3 to 4 days' incubation, while the *B. schottmulleri* cultures present a light pink or colorless coagulum. This separation has been found to parallel the differentiation of these 2 groups by agglutinin absorption tests. *B. enteritidis* is similar in its reaction to the animal para B cultures and in contrast to *B. schottmulleri*.

# THE PRODUCTION OF HYDROCYANIC ACID BY BACILLUS PYOCYANEUS

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Emerson, Bailey, and Cady noted the formation of hydrocyanic acid in decaying protein.<sup>1</sup> Clawson and Young in a series of qualitative experiments reported that a number of different strains of bacteria produce hydrocyanic acid.<sup>2</sup> In addition to these reports Kendall, Day and Walker included *B. pyocyaneus* with other bacteria in some of their studies on bacterial metabolism.<sup>3</sup>

The purpose of this work was (1) to determine the optimum reaction for the production of HCN by strains of *B. pyocyaneus*; (2) to devise a quantitative method of determining the amount of HCN produced; (3) to determine the effect of anaerobic growth; (4) to determine whether HCN is produced by an extracellular enzyme; (5) to determine any correlation between the amount of HCN, pigmentation, and rapidity of liquefaction of gelatin; (6) to determine relative amounts of HCN produced in gelatin, broth, eggwhite, whole egg, and synthetic broth; (7) to note the effect on the amount of HCN of non-HCN-producing organisms; (8) to see if *B. pyocyaneus* will produce HCN in the animal body after death, as this might be of some medico-legal importance.

Nine strains of *B. pyocyaneus* were used in the experiments, all of which were known cultures from reputable sources, as follows: No. 68 from the American Museum; 184 from the University of Kansas; 174, 175, 176, 177, 178, and 180 from the University of Chicago and Rush Medical College, and 179 from the University of Michigan.

The determination of hydrogen-ion concentration of mediums was made by the colorimetric method with buffers according to Cole.<sup>4</sup>

Qualitative tests for HCN were made by the Shoenbein or guaiac copper method, which is open to many objections and can be used only as a preliminary test. The quantitative estimation was carried on as follows: The organisms were grown in medium contained in 250 cc Erlenmeyer flasks, fitted with 2 hole stoppers, and air, filtered through soda lime and cotton, was aerated through the flasks into 4% KOH solution. At the end of 7 days the KOH solution was added to the medium, the whole made acid with sulphuric acid and distilled into 4% KOH. One cc yellow ammonium sulphide was added to

Received for publication, Feb. 28, 1921.

<sup>1</sup> Practical Physiologic Chemistry, 1919, p. 24.

<sup>2</sup> Ibid., p. 419.

<sup>3</sup> J. Am. Chem. Soc., 1914, 36, p. 1937.

<sup>4</sup> Biol. Chem., 1919, 40, p. 243.

the distillate, and the whole evaporated to dryness on a water bath. The residue was extracted with three 10 cc portions of acetone, and the acetone evaporated off and water added. Fifteen drops of 5% ferric chloride solution were added to the potassium sulphocyanate solution, and the color compared to that produced by a standardized solution of KSCN: 1 cc equals 0.01 mg. HCN.

Anaerobic conditions were obtained by growing the organisms in bottles which were sealed into a large jar containing sodium hydroxide solution and pyrogallol.

In order to find whether HCN is produced by an extracellular or intracellular enzyme, broth culture of *B. pyocyaneus* was filtered through a Berkefeld filter and the filtrate allowed to stand in gelatin for 7 days, when the HCN content was determined.

To learn whether HCN is produced in the animal body, 5 cc of an 18-hour old broth culture of *B. pyocyaneus* were injected intravenously into each of 4 rabbits, which were killed in a few hours. The liver, spleen, and stomach of each were distilled from acid solution into 4% KOH and subsequently tested for HCN. The rabbits were allowed to lie in an ice box at 10 C. until the distillations were made.

TABLE 1  
VARIATION OF HCN DUE TO HYDROGEN-ION CONCENTRATION

Strain	20% Whole Egg Broth, P <sub>H</sub> 5.6, Percentage	20% Egg White Broth, P <sub>H</sub> 6.8, Percentage	Gelatin, P <sub>H</sub> 5.8, Percentage	Gelatin, P <sub>H</sub> 6.8, Percentage	Synthetic Broth, P <sub>H</sub> 7, Percentage
68	0.127	0.0043	0.00686	0.00183	0.0125
184	0.113	0.0157	0.00688	0.00309	0.0145
174	0.0069	0.0041	0.00064	0.0028	0.050
175	0.0577	0.0098	0.0016	0.0101	0.0135
176	0.0115	0.0059	0.00141	0.00064	0.040
177	0.173	0.0075	0.0081	0.0114	0.0085
179	0.0052	0.0046	0.000686	0.00081	0.0035
180	0.0298	0.0051	0.00297	0.00064	0.030
178	0.0035	0.0041	0.00066	0.000686	0.075

The first distillation was made 12 hours, the second 24 hours, the third 48 hours, and the fourth, 7 days after death. Three controls were carried along simultaneously. On one the distillation was made at death, and on the other two, 7 days after death, one having been kept intact at 10 C. and the other operated on, and the liver, spleen, and stomach removed under sterile conditions and preserved at 10 C.

The medium used in this work was made according to formulae in "Standard Methods of Water Analysis, 1917." The synthetic broth is essentially that used in the methyl red test.

Variation of HCN due to hydrogen-ion concentration is given in table 1, which gives HCN % of total nitrogen.

Most strains yield more HCN at P<sub>H</sub> 5.6 to 5.8. Cultures 174 and 178, strong acid producers, appear to produce HCN better at lower hydrogen-ion concentration, while 68 and 180, unusually strong alkali producers, show a decrease of HCN in medium of lower hydrogen-ion concentration.



The presence of oxygen plays an important part in HCN production as shown in table 2.

Little HCN is produced anaerobically and the amount is practically constant for the change in  $P_H$ . A plain broth culture of *B. pyocyaneus*, preserved anaerobically for 16 months, was found to be alive and a good green pigment producer when transferred to agar, but no HCN was found in the broth.

TABLE 2  
RESULTS WITH STRAIN 184 BOTH IN MMG. OF HCN AND HCN PERCENTAGE OF  
TOTAL NITROGEN

	Gelatin, $P_H$ 5.8		Gelatin, $P_H$ 7.8	
Aerobic.....	0.335 mmg.	0.00773%	0.100 mmg.	0.00228%
Anaerobic.....	0.0321 mmg.	0.000739%	0.0322 mmg.	0.000740%

In the gelatin, to which 1 c.c. of sterile filtrate from a pyocyaneus broth culture was added, no HCN was produced although the gelatin was liquefied.

There is a certain correlation between the amount of HCN, pigmentation and liquefaction, as shown in table 3.

TABLE 3  
CORRELATION BETWEEN AMOUNT OF HCN, PIGMENTATION AND LIQUEFACTION

Strain	Pigmentation	Gelatin Liquefaction	Mg. HCN in Gelatin, $P_H$ 5.8
68	Dark green	Good	0.30
184	Dark green	Good	0.335
174	Brown	Slow	0.028
175	Green	Good	0.070
176	Green	Good	0.050
177	Light green	Good	0.350
178	Brown	Slow	0.029
179	Green	Good	0.030
180	Dark green	Good	0.130
Blank	.....	.....	0.028

Strains 174 and 178, brown pigment producers, were slow in liquefying gelatin and at the end of 7 days the cool gelatin cultures poured from the flasks with difficulty, while all the others were a thin liquid. The two organisms did not produce a measureable amount of HCN. However, pigmentation and HCN production are nevertheless independent functions, for lowering the HCN formation by lowering the hydrogen-ion concentration does not diminish pigment production.

TABLE 4  
RELATIVE PERCENTAGES OF HCN PRODUCED IN VARIOUS MEDIA EXPRESSED AS HCN%  
OF TOTAL NITROGEN

Strain	20% Egg White Broth, Percentage	20% Whole Egg Broth, Percentage	Gelatin, Percentage	Synthetic Broth, Percentage
68	0.0043	0.1270	0.00686	0.0125
184	0.0157	0.1130	0.00688	0.0145
174	0.0041	0.0069	0.0094	0.0500
175	0.0098	0.0577	0.0016	0.0135
176	0.0059	0.0115	0.00114	0.0400
177	0.0075	0.1720	0.0081	0.0085
178	0.0041	0.0035	0.00066	0.0750
179	0.0046	0.0052	0.000686	0.0035
180	0.0051	0.0298	0.00297	0.030
Blank	0.0041	0.0035	0.00064	0.0035

The quantity of HCN ranges from a mere qualitative test to 0.173% of the total available nitrogen. Whole egg broth 20% seems to be the most favorable medium for HCN formation and gelatin is about the least productive, due in part at least to the fact that not all the gelatin is immediately acted on.

TABLE 5  
THE EFFECT ON HCN PRODUCTION OF A NON-HCN-PRODUCING COMBINATION IN  
MG. OF HCN.

Strain	Gelatin Original Culture, Mg.	Gelatin, Culture Recovered from Egg, Mg.
68.....	0.300	0.260
184.....	0.335	0.270
174.....	0.028	0.026
175.....	0.070	0.100
176.....	0.050	0.030
177.....	0.350	0.250
178.....	0.029	0.050
179.....	0.039	0.028
180.....	0.150	0.090
Control.....	0.028	0.028

Growth in egg white does not seem to increase the HCN producing power of the bacillus, as has been stated;<sup>1</sup> in fact most strains produce HCN better in gelatin before growth in egg white than after. The contaminating organisms introduced in egg white were gram-negative rods but not HCN producers.

The results of animal inoculation (table 6) are interesting for they show an increasing amount of HCN in the bodies of the inoculated rabbits.

TABLE 6  
RESULTS OF ANIMAL INOCULATION  
Inoculated Rabbits

1.	Distillation, 12 hours after death, 0.010 mg. HCN
2.	Distillation, 24 hours after death, 0.016 mg. HCN
3.	Distillation, 48 hours after death, 0.020 mg. HCN
4.	Distillation, 7 days after death, 0.085 mg. HCN
Controls	
1.	Distillation at death.....0.007 mg. HCN
2.	Distillation after 7 days in body...0.050 mg. HCN
3.	Distillation after 7 days sterile....0.045 mg. HCN

The 5 c c broth used for inoculation, contained 0.003 mg. HCN. It is evident from the data that a measureable quantity of HCN is produced in the viscera of a rabbit after death. Since *B. pyocyaneus* is frequently found in the human body, several strains having been recovered here from feces of influenza patients, it is reasonable to believe that in postmortem examination sufficient HCN may be produced by *B. pyocyaneus* to give a positive test for cyanide.

#### SUMMARY

The optimum reaction for the production of HCN by most strains of *B. pyocyaneus* is  $P_H$  5.4 to 5.8.

The sulphocyanate colorimetric method of estimating minute quantities of HCN is a satisfactory one, but control tests must always be made, because in protein medium there is sufficient sulphocyanate formed on distillation to give a positive reaction.

Oxygen is necessary for the production of HCN by *B. pyocyaneus*.

HCN is not produced by a filtrable extracellular enzyme.

Pigmentation, gelatin liquefaction, and HCN production, though independent functions, show a close relationship.

Whole egg broth is the most favorable medium for HCN production, but synthetic medium is second, indicating that a favorable synthetic medium may be devised.

Non-HCN-producing contaminating organisms appear to be slightly inhibitive of HCN formation.

*B. pyocyaneus* does produce a measureable amount of HCN in the animal body, which may be of some medicolegal importance.

There is a marked variation among the different strains of *B. pyocyaneus* as to the amount of HCN production.

# A MICROSCOPIC METHOD FOR ANAEROBIC CULTIVATION

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The method proposed in this paper is a modification of the usual moist chamber preparation; the anaerobic system is obtained by the absorption of oxygen by alkaline pyrogallate.

## APPARATUS

The apparatus required is simple. As may be seen from fig. 1, it consists of a moist chamber containing a cell of  $H_2O$  surrounded by a moat of alkaline pyrogallate. The preparation of the anaerobic moist chamber demands no special apparatus other than that usually found in the routine equipment of the laboratory. The large object slide,  $s$ , and large outer chamber ring,  $r_1$  (2.5x1 cm.); large coverglass,  $c$ , are the same as those used in Hansen's method of single cell cultivation of yeasts. The inner ring,  $r_2$ , is not only smaller in diameter (1.5 cm.), but also less in height (0.8 cm.) than the outer ring. If rings of the proper height (0.8 cm.) are not at hand, they may be prepared by grinding down higher rings of the same diameter, such as the 1.5x1 cm. rings usually furnished for routine moist chamber studies.

The chemicals required are pyrogallol, 5% KOH, high melting point paraffin, vaseline or stopcock grease.

## MANIPULATION

(a) The preparation of the anaerobic moist chamber is simple and involves little more than the careful sealing of the two rings to the object slides in the relative position shown in the figure.

Thorough sealing of the rings is required. It was found that the following procedure gives good results: A ring of melted paraffin is applied to the object slide by means of a brush or a match in such a way as to give a film or shallow layer of paraffin over both the area covered by the bottom of both rings and the area between them. The area circumscribed by the inner ring is kept free of paraffin. The layer of paraffin is sealed more firmly to the slide by passing a hot spatula or other instrument over the paraffin after its application. Then the small ring is heated slightly, dipped into melted paraffin and sealed to

the slide by pressing it down on the inner edge of the paraffin layer. A film of paraffin is coated over the outer side of the ring after it is sealed to the slide. Applying an excess of paraffin several times and passing a hot spatula close around both edges of the bottom of the ring after it is fastened to the slide will insure a perfect seal. The large ring is sealed to the slide in the same manner. A film of paraffin is then applied around the inner side of the large ring.

The application of the films of paraffin on the sides of the rings, together with the resealing around the edges of both rings, forms a paraffin-lined moat between the cells. If the moat is not already completely lined with paraffin, this may be accomplished by the introduction of melted paraffin into the moat by means of a capillary pipet. The paraffin lining thus obtained is desirable to obtain the perfect fastening of the rings to the slide by protecting the glass from etching by the alkali. The efficiency of sealing of the rings may be tested by careful examination or by introduction of water into the cells.

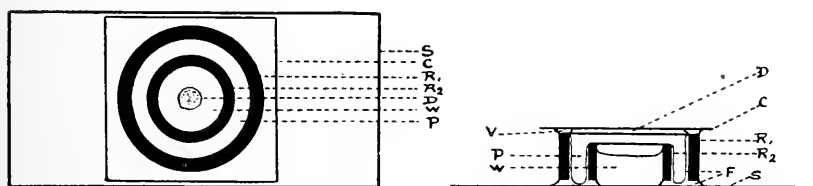


Fig. 1.—Apparatus for anaerobic cultivation (natural size). A, top view; B, cross section; S, slide; C, cover glass; R<sub>1</sub>, ring; R<sub>2</sub>, ring; D, drop culture; W, water; P, alkaline pyrogallol acid; V, vaseline; F, paraffin.

The most careful preparation of the chambers does not require 15 minutes of the worker's time. They may be used immediately, or may be prepared some time before the occasion. It is usually desirable, however, to prepare them some time before, to permit testing of the sealing.

(b) Preparation of the Anaerobic Culture: Preparation of the anaerobic moist chamber culture involves little more manipulation than that required in the preparation of the usual aerobic moist chamber culture. By means of a small spatula or folded paper, approximately 0.07 gm. pyrogallol is put into the bottom of the paraffin-lined moat. The inner cell is filled with water to a short distance from the top of the ring. The upper edge of the outer ring is then covered with stopcock grease or vaseline.

A drop of recently boiled culture medium is placed on the large cover slip held in Carnet forceps. The drop is inoculated with the anaerobe under investigation; the cover slip is then inverted. At this time 5% KOH is introduced into the moat; the inoculated cover slip is then sealed on the top of the large ring.

(c) Incubation and Observation: The anaerobic moist chamber culture so prepared is now ready for microscopic observation and incubation. The culture can be incubated on the microscope stage and be under the direct observation of the investigator throughout the history of the culture.

Anaerobic hanging block preparations could be prepared with practically the same technic.

#### DISCUSSION OF THE METHOD

Anaerobic conditions are obtained by the absorption of oxygen by the alkaline pyrogallate solution in the moat surrounding the cell of water. The amount of pyrogallol used presents an excess over that required to absorb the small quantity of oxygen present in the part of the chamber not occupied by the pyrogallate and water. The total volume of the chamber is approximately 5 c c, but the prepared chamber contains less than 2 c c free air space. These relations insure low oxygen concentrations in the prepared chamber during incubation.

The chamber moat presents approximately 3 square cm. surface area for oxygen absorption by the pyrogallate. The large surface exposure gives a rapid absorption of oxygen.

Observation of the culture is made by means of the light passing through the inner cell of water as in the case of the usual moist chamber preparation. Loosening of the inner ring, which may result in entrance of the dark pyrogallate solution into the inner cell, will interfere with the illumination of the object. This will occur in poorly prepared chambers.

Evaporation and change in concentration of the culture medium is reduced to a minimum by the saturation of the chamber with water vapor from the inner cell, which presents a large surface of water.

This anaerobic moist chamber was used successfully to study the growth and development of obligate anaerobes, namely *B. tetani* and *B. botulinus*. The photomicrograph of the culture could be made directly at different intervals. At the same time, it was demonstrated that the growth of an obligate aerobe, viz., *B. subtilis*, in the chamber, was inhibited.

## SUMMARY

By the use of this anaerobic moist chamber, the growth and development of anaerobic micro-organisms may be studied by direct microscopic observation throughout the uninterrupted history of the culture.

This microscopic method of anaerobic cultivation possesses the general advantages of oxygen absorption anaerobic methods.

In addition, it possesses the following advantages over the older microscopic oxygen absorption anaerobic methods: simplicity of apparatus, ease of manipulation, good illumination of object, high degree of oxygen absorption, rapid decrease in oxygen concentration, and minimum evaporation and change in concentration of the culture medium.

# THE PRODUCTION OF CO<sub>2</sub> BY THE TYPHOID BACILLUS AND THE MECHANISM OF THE RUSSELL DOUBLE SUGAR TUBE

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In the course of some work on the nature of the changes in the Russell double sugar medium during the growth of the typhoid bacillus, a fact of considerable theoretical interest was observed, namely, that the typhoid bacillus produces CO<sub>2</sub> in appreciable amounts. This fact has apparently escaped previous observation, but seems definite in spite of the absence of any visible evidence of gas.

The explanation of the appearance of the double sugar tube as given in the original description and repeated elsewhere, is as follows:<sup>1</sup>

"The entire point of the medium rests upon the difference in changes produced by the growth of the typhoid bacillus under aerobic and under the imperfect anaerobic conditions found in the butt of the tube, where the bacillus obtains its oxygen by breaking down the glucose with the liberation of considerable acid; on the surface, however, in the presence of free oxygen, no acid is formed."

In this explanation no place is found for the fact that the typhoid bacillus ferments glucose with acid end-products under both aerobic and anaerobic conditions. It occurred to the writer that the glucose might be fermented in the slant as well as in the butt and that the small amount of acid formed from the 0.1% glucose might volatilize and leave the medium apparently unaffected. In order to test this hypothesis several experiments were made with results which support this explanation as one important factor in the mechanism of the tube.

When a double sugar tube inoculated with the typhoid bacillus (Rawlings), is coupled up<sup>2</sup> in an anaerobic system of pyrogalllic acid and KOH, the appearance of the slant is the same as usual. In other words, in an atmosphere free of oxygen, no acid is apparently produced, as should be the case according to the original explanation. If a volatile acid is assumed, however, the neutral slant can be readily explained by absorption of the acid by the KOH.

Received for publication March 17, 1921.

<sup>1</sup> Russell, F. F.: *Jour. Med. Research*, 1911, 25, p. 227.

<sup>2</sup> Nichols, H. J., and Schmitter, F.: *Ibid.*, 1906, 15, p. 113.



In an inoculated double sugar tube covered with oil, the slant becomes strongly acid. This result, in the light of our knowledge of oil seals,<sup>3</sup> can be explained by the retention of acid better than by the exclusion of oxygen.

In an inoculated double sugar tube sealed by heat or paraffin the slant becomes acid. This result also can be explained better by assuming a retention of a volatile acid rather than an exhaustion of oxygen.

When an inoculated double sugar tube is coupled up in a tight system with an uninoculated tube, the slant of the second tube becomes acid as well as the first; in other words, some volatile acid passes over to the uninoculated tube.

Finally a set of glucose agar Andrade tubes of various strengths from 0.01% to 1% by tenths, inoculated with the typhoid bacillus, gives these results:

TABLE 1  
RESULTS OF INOCULATION OF GLUCOSE ANDRADE TUBES WITH THE TYPHOID BACILLUS

Slant Acid			Slant Acid	
			24 Hours	48 Hours
			(+)	(-)
0.01	—	0.2	+	—
0.02	—	0.3	+	—
0.03	—	0.4	+	—
0.04	—	0.5	+	—
0.05	—	0.6	+	—
0.06	—	0.7	+	—
0.07	—	0.8	+	—
0.08	—	0.9	+	—
0.09	—	1.0	+	—
0.10	—			

These results indicate that the typhoid bacillus ferments glucose in the slant under aerobic conditions. The acid products of fermentation are evident above 0.3% and were undoubtedly present in small amounts in the lower percentages, but leave no evidence of fermentation on account of their escape and neutralization. This latter additional factor is indicated by the appearance of the 0.2% glucose tube which shows some acid on the slant in 24 hours, but none in 48 hours. It is common knowledge among users of the double sugar tube that a distinct alkaline reversion occurs after several days. It has recently been shown by Ayers and Ruff<sup>4</sup> that simultaneous acid and alkaline fermentations occur with members of the colon-aerogenes group.

It is seen that the volatile acid, of which evidence has been presented, acts very much like CO<sub>2</sub>. In fact, the appearance of a litmus double

<sup>3</sup> Gates, F. L., and Olitsky, P. H.: Jour. Exper. Med., 1921, 33, p. 51.

<sup>4</sup> Jour. Infect. Dis., 1918, 23, p. 188.

sugar tube, under the varying conditions given above, can be exactly duplicated by saturating sterile medium with  $\text{CO}_2$ . But the typhoid bacillus does not produce  $\text{CO}_2$  according to any authority. The most complete work on the acid products of the growth of the typhoid bacillus in glucose is that of Harden<sup>5</sup> who found about 40% of lactic acid, 17% of acetic acid, 17.5% of formic acid, a trace of succinic acid and 10% alcohol. "He points out that with forms like *B. typhosus*, formic acid must be produced from the fractions of the molecule which with *B. coli* yield  $\text{CO}_2$  and  $\text{H}_2$ ."<sup>6</sup> As acetic and formic acids are volatile, it was at first thought that they might be responsible for the appearance of the tube. About this time, however, the writer became acquainted with the work of Ayers, now in press, on the production of  $\text{CO}_2$  by streptococci. Ayers used the little known but practical Eldredge tube,<sup>7</sup> and showed that  $\text{CO}_2$  is produced not only from sugars but also from proteins.

TABLE 2  
RESULTS OF EXPERIMENTS WITH ELDRIDGE TUBE

	Gm. of $\text{CO}_2$
Control, uninoculated, 1% glucose extract broth.....	0.0
Uninoculated broth + 1 c c formic acid.....	0.0066
Uninoculated broth + 1 c c acetic acid.....	0.0035
Uninoculated broth + 1 c c sodium bicarbonate.....	0.0158
Plain extract broth (average of 2 tests).....	0.011
Glucose extract broth (average of 5 tests).....	0.0178
1% Witte peptone water.....	0.0019
1% peptone + 1% glucose.....	0.0056
0.25% beef extract.....	0.0082
Sugar free broth.....	0.0044
Sugar free broth + peptone.....	0.0059
Sugar free broth + peptone + glucose.....	0.0127

#### THE PRODUCTION OF $\text{CO}_2$ BY THE TYPHOID BACILLUS

Some experiments with the Eldredge tube yielded the following significant results. (In all the experiments 50 c c of medium and the Rawlings typhoid strain were used, 25 c c of 0.1 N  $\text{Ba}(\text{OH})_2$  were placed in the absorbing tube. After 24 hours' growth, this was titrated with 0.1 N  $\text{HCl}$  to a loss of color with phenolphthalein and then to a pink color with methyl orange. The difference between 25 and the first figure [for example 17.4], such as 7.6 gives a result in terms of 0.1 N,  $\text{H}_2\text{CO}_3$ . This figure was in turn multiplied by 0.0022 to give gm., of  $\text{CO}_2$ ).

This table shows that  $\text{CO}_2$  is produced by the typhoid bacillus both from glucose and from proteins. It also shows that the decomposition of formic acid is not the source of much of the  $\text{CO}_2$ . The relative

<sup>5</sup> Jour. Chem. Soc., 1901, 79, n. 610.

<sup>6</sup> Winslow, Kligler and Rothberg: Jour. Bacteriol., 1919, 4, p. 429.

<sup>7</sup> Eldredge, E. E., and Rogers, L. A.: Centralbl. f. Bakteriöl., 1914, 40, p. 5.

amount of CO<sub>2</sub> to other acids is of importance and incidentally has a direct bearing on the explanation of the double sugar tube. In terms of 0.1 N acid and alkali, the ratio was found to be 7.6 to 9.5 or about 45% of the total acid produced is CO<sub>2</sub>.

CO<sub>2</sub> is soluble in about 60% its own volume in water at 760 mm. of Hg and 35 C. and the fallacy of the ordinary CO<sub>2</sub> determination has been pointed out by Keyes<sup>8</sup> and Clark.<sup>9</sup> It is evidently necessary to use more exact methods such as those of Harden, Keyes, Rogers, Clark and Davis,<sup>10</sup> and Eldredge and Rogers. Just why the CO<sub>2</sub> was missed in Harden's work on the typhoid bacillus, and by other observers, the writer is unable to say. Nor can he discuss the physical chemistry of CO<sub>2</sub>. But if the Eldredge is reliable, as it seems to be, there is no doubt about the production of CO<sub>2</sub> in important amounts. Several other organisms, such as paratyphoid A and B, the dysentery bacilli and *B. subtilis*, affect the double sugar mediums in somewhat the same way as the typhoid bacillus. All these organisms yield considerable amounts of CO<sub>2</sub> in the Eldredge tube from dextrose broth.

The explanation of the double sugar tube is largely an academic question and does not affect the value of this medium, which with slight variations in indicators or sugar content has come into routine use in the diagnosis of organisms of this group. But, of course, the most truthful explanation will link up best with other observations and will be least confusing to students.

#### SUMMARY

CO<sub>2</sub> is produced by the typhoid bacillus in significant amounts both from sugars and from proteins.

The appearance of the Russell double sugar tube during the growth of the typhoid bacillus is not due to direct oxygen requirements. It is due (a) to the retention of CO<sub>2</sub> in the butt of the tube and its escape from the slant and (b) to alkaline reversion of other acids.

<sup>8</sup> Jour. Med. Res., 1909, 21, p. 69.

<sup>9</sup> Science, 1913, 38, p. 669.

<sup>10</sup> Jour. Infect. Dis., 1914, 14, p. 411.

# EFFECTS OF FILTRATION ON THE POTENCIES OF ANTITOXINS

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The object of the work was to ascertain whether any antitoxic units were adsorbed when a product, such as tetanus antitoxin, was filtered through a Berkefeld type filter. Mechanical losses were not considered.

The need of comparing the filtered with original products was pointed out some time ago, by the Chief of the Bureau of Animal Industry, Dr. John R. Mohler.

*Materials.*—Table 1 briefly describes the antitoxic products used. More than 390 guinea-pigs were used in the inoculation tests, carried out by the official methods<sup>1</sup> of standardizing tetanus and diphtheria antitoxins with slight modifications.<sup>2</sup> In table 2 an attempt is made to show the practical relations between toxins and standard antitoxins.

*Apparatus.*—Only such flasks, delivery pipets and capacity pipets as were tested and approved by the U. S. Bureau of Standards were used. For the detection of slight changes in potency, i.e., a decrease of 10% of the original, 1 c c pipets graduated to 0.01 c c, must be used with an error of less than 0.01 c c. Syringes of the Record type were tested, before use, by weighing the water delivered.

*Filtration.*—Twenty gm. of diatomaceous earth were mixed with 60 c c of antitoxic product and let stand for varying times. The pasty mixture was then packed around a small Mandler filter, previously sterilized, and filtration was carried out as usual, avoiding evaporation, etc. The numbers of antitoxic units per c c of filtered and original product were compared by the official method of guinea-pig inoculation (see table 4).

*Modifications of the Official Technic.*—The following departures from the official methods of standardizing tetanus and diphtheria antitoxins were found convenient: (a) Instead of preparing one dose in one syringe for one guinea-pig injection, prepare 2 or 3 times the desired

Received for publication March 12, 1921.

<sup>1</sup> Rosenau, M. J.: Hygienic Lab. Bull. 21, 1905. Rosenau, M. J., and Anderson, J. F.: Hygienic Lab. Bull. 43, 1908.

<sup>2</sup> Berg, W. N., and Kelser, R. A.: Jour. Agric. Research, 1918, 13, p. 471.

dose in a sterile, glass stoppered weighing bottle. (b) When injection is made, use a syringe of the Record type, measuring the dose for injection by pushing the plunger from one graduation to another and not from one graduation mark to the end of the barrel. (c) Instead of injecting the dose in a final volume of 4 c c, inject it in a volume of 2 c c.

*Chemical Analyses.*—Adsorption of protein was looked for in the products injected into guinea-pigs. Determinations were made of (1) total solids, (2) ash, and (3) coagulable protein. The chemical methods have been previously described.<sup>3</sup> The analyses are summarized in tables 3 and 4.

#### SUMMARY

In every one of 13 filtration experiments with diatomaceous earth, protein was adsorbed from tetanus and diphtheria antitoxic products in quantities ranging from 5 to 33% of the original protein content. In 4 adsorption experiments with fullers' earth, the protein adsorbed was 40 to 78%, depending on conditions.

When the experimental conditions were such that protein adsorption was slight, i.e., 10 to 15%, an adsorption of antitoxic units was not detected.

When protein adsorption was high, i.e., 20 to 78% of the total protein, a corresponding adsorption of 8 to 66% of the original tetanus antitoxic units was detected.

The results indicate that: A rapid laboratory method of testing whether a filter system adsorbs antitoxic units is to estimate, by any convenient chemical method, coagulable protein or solids-not-ash in the original and the filtered product. If there is protein adsorption in large amount, (20% or more), there will be a detectable adsorption of antitoxic units. If protein adsorption is less than 20%, there may be no detectable adsorption of antitoxic units.

A rapid, accurate method of standardizing antitoxins in vitro is much needed, even if only preliminary values are obtained.

It is doubtful whether two workers in separate laboratories would obtain values within 10% of each other in the standardization of the same product by the official method.

As ordinarily carried out, filtration of an immune serum or similar product through a Berkefeld type filter does not result in appreciable losses of antitoxic units.

<sup>3</sup> Berg, W. N.: Jour. Lab. and Clinic Med., 1921, 6, p. 223.

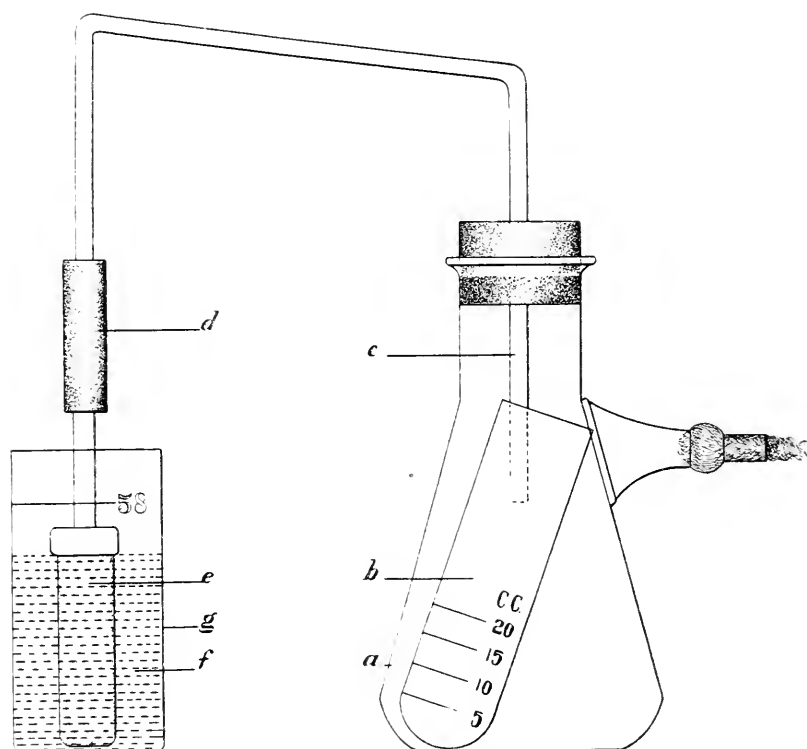


Fig. 1.—a, filter flask capacity 250 c.c.; b, graduated tube to receive filtrate; c, glass connecting tube; d, heavy rubber tube connection between c and e, small Berkefeld filter; f, pasty mixture of diatomaceous earth, and antitoxic product 60 c.c.; g, glass weighing bottle, stopper not shown.

TABLE 1  
DESCRIPTION OF ANTITOXIC PRODUCTS

Designation of Product	Maker	Potency Stated by Maker in Units per C c	When Obtained	Potency Determined by Writer in Units per C c	Remarks by Maker
Tetanus antitoxin 420 F	Lederle	About 300	December, 1916	300 (1917) 250 (1920)	Berkefeld filtered by maker
Tetanus plasma 9588	Mulford	About 200	March, 1920	500 to 540	Citrated plasma, not filtered, preserved with tricrosol
Tetanus serum 2030	Lederle	250 to 300	April, 1920	320 to 340	Serum not filtered, contains no citrate or oxalate, preservative, 0.35% cresol
Standard tetanus antitoxin T 23, T 24	U. S. Hygienic Laboratory	5.0	April, 1920		
Diphtheria antitoxin 615 LN	Lederle	About 800	December, 1916	900 to 1000	Berkefeld filtered
Diphtheria plasma 9004	Mulford	About 400	April, 1920	700 to 750	Citrated plasma, not filtered, preserved with tricrosol
Diphtheria serum 2401	Lederle	250 to 300	April, 1920	400 to 450	Not filtered, contains no citrate or oxalate; chloroform 0.4 %
Standard diphtheria antitoxin B 142, B 144, B 145	U. S. Hygienic Laboratory	6.0	March, 1920		

TABLE 2  
RELATIONS BETWEEN TOXINS AND STANDARD ANTITOXINS

	Tetanus	Diphtheria
Practical relation between test doses	When each of 4 guinea-pigs is subcutaneously injected with 0.1 unit of antitoxin mixed with 1 test dose of toxin, 3 should die between 72 and 96 hours; 1 close to 96 hours	When each of 4 guinea-pigs is subcutaneously injected with 1 unit of antitoxin mixed with 1 test dose of toxin, 3 should die between 72 and 96 hours; 1 close to 96 hours
Standard antitoxin from Hygienic Laboratory	Glycerolated solution 1 c c contains 5 units	Glycerolated solution 1 c c contains 6 units
Standard weight of guinea-pig...	350 gm.	250 gm.
Test dose of antitoxin injected into 1 guinea-pig	0.1 unit	1 unit
Test Dose (L+ dose) of toxin injected into 1 guinea-pig	0.00075 gm.	0.265 c c
Toxin, from Hygienic Laboratory	Powder	Liquid
Number of minimal lethal doses (MLD) in one test of L+ dose of toxin	100 approximately	201 theoretically,* variable practically

\* Hygienic Laboratory Bulletin 21, p. 29.

TABLE 3  
ANALYSES OF ANTITOXIC PRODUCTS

Product	Experiment No.	Solids Not Ash in 100 C c		Solids Not Ash Adsorbed, Percentage	Ash in 100 C c		Total Time of Filtration, Hours
		Original, Gm.	Filtered Through Diatomaceous Earth and Berkefeld, Gm.		Original, Gm.	Filtered Through Diatomaceous Earth and Berkefeld, Gm.	
Normal horse serum 2	..	7.86	7.52	4.3	0.780	2.075	..
Tetanus antitoxin 420 F	2	6.74	5.55	17.7	0.68	0.69	2
	3	6.71	4.30	35.9	0.65	1.16	46
Tetanus plasma 9588	4	10.03	9.06	9.7	0.98	1.06	3 $\frac{1}{4}$
	7	9.90	9.14	8.9	0.93	1.06	24
Tetanus serum 2030	5	8.56	7.85	8.3	0.77	0.70	3 $\frac{1}{2}$
	6	8.66	7.69	11.2	0.70	0.80	26
Diphtheria serum 2401	21	7.64	7.06	7.6	0.71	0.79	3
	32	....	6.61	13.5	....	0.76	44
Diphtheria plasma 9004	28	9.50	8.74	8.0	0.80	0.95	2 $\frac{1}{2}$
	31	....	8.64	9.1	....	0.96	48 $\frac{1}{2}$
Diphtheria antitoxin 615 LN	27	12.27	11.54	5.9	0.91	0.97	6 $\frac{1}{2}$
	30	....	11.74	4.3	....	1.08	77

TABLE 4  
ANALYSES OF ANTITOXIC PRODUCTS

Product	Experiment No.	Coagulable Protein in 100 C c		Coagulable Protein Adsorbed, Percentage	Antitoxic Units Adsorbed, Percentage	Total Time of Filtration, Hours
		Original, Gm.	Filtered Through Diatomaceous Earth and Berkefeld, Gm.			
Tetanus antitoxin 420 F	1	6.35	5.73	9.8	..	(few minutes)
	2	....	....	....	8	2*
	3	6.11	4.07	33.3	14	46
Tetanus plasma 9588	4	8.96	8.30	7.4	0	3 $\frac{1}{4}$
	7	8.96	8.16	8.9	0	24
Tetanus serum 2030	5	7.98	7.22	9.5	0	3 $\frac{1}{2}$
		8.02	7.42	7.5		
	6	8.02	7.14	11.0	0	26
Diphtheria serum 2401	21	7.14	6.66	6.7	0	3
	32	....	6.30	11.8	0	44
Diphtheria plasma 9004	28	8.86	8.23	7.1	0	2 $\frac{1}{2}$
	31	....	8.10	8.6	0	48 $\frac{1}{2}$
Diphtheria antitoxin 615 LN	27	12.12	11.53	4.9	0	6 $\frac{1}{2}$
	30	....	11.24	7.4	0	77

\* See table 3 for analytic data.



# OBSERVATIONS ON THE SPREAD AND PERSISTENCE OF THE HEMOLYTIC STREPTOCOCCI PECULIAR TO SCARLET FEVER

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## 1. THE STREPTOCOCCI ISOLATED FROM ATTENDANTS AND SURROUNDINGS OF SCARLET FEVER PATIENTS

In previous articles I<sup>1</sup> have shown that the hemolytic streptococci isolated from early cases of scarlet fever and its complications belong to a distinct biologic group, apparently peculiar to scarlet fever, on account of their being specifically opsonified and agglutinated by the serum of a sheep immunized with a hemolytic streptococcus from scarlet fever. This serum also specifically protected mice against hemolytic streptococci from scarlet fever. Bliss,<sup>2</sup> reached similar conclusions from agglutination tests of streptococci from scarlet fever with immune rabbit serum as did also more recently Gordon.<sup>3</sup>

It seemed interesting to determine by opsonic and agglutination tests whether hemolytic streptococci from the rooms, eating utensils and attendants of diphtheria and scarlet fever patients belong to this same biologic group, and tests have been made of 20 strains of hemolytic streptococci isolated by Dr. W. J. Matousak<sup>4</sup> from the scarlet fever and the diphtheria rooms of the Durand Hospital. His results, reported elsewhere, were in brief as follows:

Hemolytic streptococci were isolated from the walls of diphtheria rooms once and from the nurses' shoes three times, but not from the fingernails, floor, door knobs or soap brushes.

Cultures were taken in rooms of scarlet fever patients, the throats of whom (in one instance also a leg abscess) harbored hemolytic streptococci specific for scarlet fever. Hemolytic streptococci were isolated from the fingernail of a nurse once, the outside of a face mask twice, the floor three times, the floor just outside once, the wall twice, the shoes of nurses three times, the cup, fork or spoon used by patients four times. The cultures from the door knobs, soap brushes, unused dishes and light buttons were negative.

All of the 20 strains of streptococci grew in chains in broth and produced a wide zone of hemolysis, from 2 to 4 mm., on goat blood agar plates after 24 hours' incubation. The strain from the fingernail

Received for publication March 19, 1921.

<sup>1</sup> J. A. M. A., 1920, 74, p. 1386, and 75, p. 1339.

<sup>2</sup> Bull. Johns Hopkins Hosp., 1920, 31, p. 173.

<sup>3</sup> Br. Med. J., 1921, 1, p. 632.

<sup>4</sup> Jour. Am. Med. Assn., 1921, 76, p. 1490.

produced large mucoid colonies, the others were small, round and grayish. All of the streptococci fermented lactose and salicin, but not mannite or inulin, and consequently would be classed as *Streptococcus pyogenes*.<sup>5</sup>

Opsonic and agglutination experiments were made with these strains of streptococci and the serum of a sheep which had been immunized with a hemolytic streptococcus isolated from the throat of a severe acute case of scarlet fever. Immunization had been carried on 8 months when these experiments were commenced. Some of the opsonic experiments were made first with fresh immune sheep serum. The strains were tested again with serum which had been stored for six months in the ice box and was still specific for hemolytic streptococci from scarlet fever. The agglutination experiments were almost all made with the stored serum.

For the opsonic experiments, the cocci were grown on blood agar for 24 hours and suspended in physiologic sodium chlorid solution. Normal sheep leukocytes, collected in 0.2% sodium citrate solution and washed once in salt solution were used. The serum, normal and immune, was heated for one-half hour at 56 C. to remove the thermolabile element, and then diluted with salt solution. The mixtures of diluted serum, leukocytes and coccal suspensions, equal parts, were incubated for 25 minutes, smears stained with carbolthionin, 50 polymorphonuclear leukocytes counted, and the number of cells taking part in phagocytosis noted. The point of opsonic extinction was determined by finding the dilution at which opsonification ceased. When the old stored serum was used it was necessary to activate it by fresh sheep serum, one part of fresh serum being added to one part each of diluted serum, leukocytes and coccal suspension.

In the agglutination tests cocci were grown from 24 to 48 hours in ascitic dextrose broth, one part of ascitic fluid to four parts of 1.0 % dextrose broth,  $P_H$  7.8. The cultures were centrifuged, the supernatant fluid removed and the cocci washed once or twice in plain meat extract broth,  $P_H$  7.8, and finally suspended in this medium. In the tests the serum dilutions were made with plain broth, the dilutions running from 1:10 to 1:800, and equal parts of bacterial suspension were added to each tube of diluted serum, and the mixture incubated for one hour at 55 C. Tubes containing suspended cocci in broth but without serum and also mixtures of cocci in normal sheep serum diluted from 1:10 to 1:400 or higher were included in each

<sup>5</sup> Holman, W. L.: Jour. Med. Res., 1916, 34, p. 377.

test. The immunizing streptococcus was tested in each experiment to serve as a standard. Later salt solution was substituted for the broth in making the dilutions and suspending the cocci and gave somewhat clearer agglutination. One lot of ascitic fluid with a specific gravity of 1.020 caused spontaneous clumping of the cocci and could not be used.

The 4 strains isolated from the shoes of nurses and the wall of the diphtheria room were neither opsonified nor agglutinated by the immune serum in higher dilutions than by normal sheep serum. Only 5 of the 16 strains isolated in the scarlet fever rooms were found to belong to the scarlet fever group of streptococci. One of these strains was isolated from a face mask and the others from the eating utensils (fork, spoon and cup) used by scarlet fever patients harboring hemolytic streptococci, which were specific for scarlet fever. The point of opsonin extinction for these streptococci varied from 1:40 to 1:240, the point of extinction for normal sheep serum being 1:3. The lower points of extinction were found when the stored serum was used. These streptococci were agglutinated at a dilution of 1:200 to 1:400. None were agglutinated by normal sheep serum.

These experiments indicate the value of face masks as a protection from pathogenic bacteria and the necessity of careful sterilization of eating utensils used by patients with infectious diseases. The results are in accord with those of Brown, Petroff and Pesquera,<sup>6</sup> Cummings, Spruit and Reuter,<sup>7</sup> and Saelhof and Heinekamp,<sup>8</sup> who came to the conclusion that eating utensils are a source of danger in the transference of tubercle bacilli and other pathogenic bacteria, unless properly cleansed. Cummings and his coworkers found that 87% of the eating utensils used by patients or healthy carriers of hemolytic streptococci were still contaminated with this organism after they were hand washed. Saelhof and Heinekamp isolated hemolytic streptococci, virulent for rabbits, from restaurant table ware in 6.35% of the articles examined.

## 2. ATYPICAL CASE OF SCARLET FEVER

I have isolated hemolytic streptococci (*St. pyogenes*) from the throats of 10 attendants at the Durand Hospital who had tonsillitis; the throats of 3 normal nurses, one a diphtheria nurse, the other two scarlet fever nurses; from the infected fingers of two interns; and from the cerebrospinal fluid in a case of meningitis following tonsillitis.

<sup>6</sup> Thirty-Fifth Annual Med. Report, Trudeau Sanatorium, 1919.

<sup>7</sup> The Military Surgeon, 1920, 2, p. 592.

<sup>8</sup> Am. Jour. Public Health, 1920, 10, p. 704.

Only 2 of these strains were opsonified and agglutinated by the immune sheep serum, the point of opsonic extinction being 1:80 and agglutination positive at 1:400 for both strains. There was neither opsonification nor agglutination with normal sheep serum. One strain was isolated from the throat of a maid with acute tonsillitis without an exanthem; the other from a scarlet fever nurse with a very red throat, a leukocytosis of 12,500, but no eruption and no rise in temperature. The nurse had had scarlet fever previously. These 2 cases would appear to be in the same class as 2 described by Bliss, who isolated hemolytic streptococci specific for scarlet fever from 2 patients exposed to scarlet fever, who had acute tonsillitis without exanthem.

Agglutination experiments with hemolytic streptococci isolated from patients with rash suggestive of scarlet fever have proved helpful in several cases in verifying a diagnosis of scarlet fever. A streptococcus from the discharging ear of a colored boy, entering the hospital with a scarcely visible eruption, was strongly agglutinated by the immune serum. A streptococcus from the throat of a nurse with an exanthem was not agglutinated and the rash was subsequently ascribed to antitoxic serum given 5 days previously.

A streptococcus from a patient with tonsillitis and a slight erythema was not agglutinated; further examination of the patient and the course of the disease indicated that the patient did not have scarlet fever. A streptococcus isolated from the discharging ear of a diphtheria patient was agglutinated; diffuse exanthem persisting for 5 days with subsequent desquamation accompanying the urticaria following the administration of antitoxin, indicated that the patient had scarlet fever also. The nurse in attendance contracted scarlet fever 3 days after she commenced nursing this patient.

A streptococcus isolated at necropsy from the lung of a measles patient with bronchopneumonia was agglutinated by the immune serum. It was considered that this patient probably had scarlet fever on account of the discharging ears, enlarged cervical glands, purulent infection of the accessory sinuses and a diffuse erythematous eruption, accompanying the measles rash.

Two cases are of interest because the agglutination experiments throw some light on the etiologic relation between the streptococci and the pathologic conditions. In one instance the hemolytic streptococci isolated from a vaccination wound of a patient with scarlet fever were not agglutinated, indicating that the two infections were coincident; in the other case, the hemolytic streptococci isolated from the

throat and lochia of a case of puerperal fever and scarlet fever were both agglutinated in high dilutions by the immune serum, and suggested that the same streptococcus was responsible for both infections.

In my previous experiments I found that hemolytic streptococci, peculiar to scarlet fever, generally disappeared from the throats of scarlet fever patients during the third and fourth weeks of the disease, and I concluded that the streptococci isolated from the throats at the onset of the attack were immunologically different from most of those obtained during convalescence. Further studies along this line seemed necessary. When possible, 4 colonies of hemolytic streptococci (*Str. pyogenes*) were isolated from the throat of the scarlet fever patient every other day for 25 days and tested for opsonins and agglutinins with the immune sheep serum. All of the strains isolated up to the 13th day of the disease were opsonified and agglutinated in high dilutions. On the 17th day one strain was isolated which was opsonified by the immune serum, the agglutination test not being made. The other strains isolated after the 13th day were neither opsonified nor agglutinated.

### 3. PERSISTENCE OF STREPTOCOCCUS PECULIAR TO SCARLET FEVER IN THE THROAT, EARS AND NOSE

In the original group of cases reported, 2 strains of hemolytic streptococci were isolated from patients with otitis media following scarlet fever in the 4th and 6th weeks. Neither strain belonged to the scarlet fever group of hemolytic streptococci. Since then another strain has been isolated from an ear, beginning to discharge 3 weeks after the onset of a case of probable scarlet fever. Three weeks later a hemolytic streptococcus was isolated and found not to belong to the scarlet fever group, being neither agglutinated nor opsonified by the immune sheep serum.

A strain of hemolytic streptococcus has been isolated during the 6th week from the purulent discharge of the nose of a scarlet fever patient, and was specifically agglutinated at a dilution of 1:200.

Streptococci from the discharging ear of a diphtheria patient, who had scarlet fever one month previously, was markedly agglutinated by the immune serum, which indicated that the ear infection was scarlatinal in origin.

These experiments suggest that while streptococci peculiar to scarlet fever are present as a rule from 3 to 5 weeks after the onset of the disease, they may persist much longer in patients with discharges, and that they disappear at the time the patient becomes non-infectious, according to clinical experience.

## CONCLUSIONS

Hemolytic streptococci may be isolated from the floor and walls of rooms occupied by patients with scarlet fever and diphtheria, and from the fingernails, face masks and shoes of the attending nurses and from the eating utensils used by patients harboring hemolytic streptococci. Only 5 of 20 strains thus isolated were opsonified and agglutinated by the serum of a sheep immunized with a hemolytic streptococcus from scarlet fever and hence to be considered as specific for scarlet fever. Four of these strains were isolated from the eating utensils of scarlet fever patients and one from the face mask of the nurse in attendance. These results indicate the value of face masks and the necessity of disinfection of eating utensils used by patients with infectious diseases.

It would appear also that persons associated with scarlet fever patients may develop tonsillitis without an exanthem and harbor hemolytic streptococci which belong to the same biologic group as those isolated from typical cases of scarlet fever.

Agglutination of hemolytic streptococci from suspected cases of scarlet fever, by immune sheep serum specific for streptococci from scarlet fever, has proved helpful in diagnosis.

These results suggest, further, that while patients with scarlet fever generally rid themselves of hemolytic streptococci specific for scarlet fever in from 3 to 4 weeks, patients with discharges may retain them much longer, and that the streptococcus specific for scarlet fever disappears at the time when the patient becomes noninfectious, according to clinical experience.

# RECOVERY FROM RABIES, WITH REPORTS OF CASES OF TREATMENT PARALYSIS AND OF RECOVERY OF ANIMALS APPARENTLY RABID

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Notwithstanding the occasional reports of natural recoveries from rabies, this disease is generally considered to be invariably fatal. This has no doubt led to some errors in diagnosis, and persons have undoubtedly been exposed to rabies infection without being given the protection of preventive inoculation, while others developing the disease have been given doses of sedatives to control the symptoms, which might in themselves prove fatal. In fact in our own experience we have known of patients receiving doses of sedatives which might easily have been the cause of death.

Unfortunately, owing to obvious difficulties, few of the reported cases of recovery either from natural or experimental infection with rabies virus have been confirmed by subsequent laboratory investigation. Opportunities for such a study are rare. We can find in the literature no cases of recovery after natural infection in man or animals in which the diagnosis was confirmed. In our own experience we have had the three histories (included in this article) given by veterinarians but in each case too long after recovery to permit of confirmatory tests. We now report in detail the study of two cases in animals, and observations on treatment paralysis.

## TREATMENT PARALYSIS

This comparatively rare incident of antirabic treatment is made manifest by symptoms that vary in severity from those of a slight sensory neuritis to those of an acute ascending paralysis, which may end fatally. These symptoms usually appear from a week after commencement of treatment to shortly after its completion and occur most often in adults.

Levy<sup>1</sup> reports one case in which they first manifested themselves 73 days after treatment. Fielder<sup>2</sup> gives the total number of reported cases up to 1916 as 142, with 24 deaths (16.2%). Since then additional reports have been published by Mejio,<sup>3</sup> 24 cases, 4 of which were fatal; Geiger,<sup>4</sup> 8 cases of paralysis and 7 of neuritis; Levy,<sup>1</sup> 1 case; Rochaix and Durand,<sup>5</sup> 1 case; Wirschubski,<sup>6</sup> 1 fatal case; von Dziembowski,<sup>7</sup> 1 case; Papamarku,<sup>8</sup> 9 cases; Pfeiffer,<sup>9</sup> 1 case; Price,<sup>10</sup> 1 case.

To show the relative frequency of its occurrence under different methods of antirabic treatment, Fielder gives the following summary from Simon:

Method	No. of Cases Treated	Cases of Paralysis	Proportion
Classical Pasteur .....	32,676	6	1:5446
Modified Pasteur .....	8,657	16	1:541
Högyes .....	51,417	3	1:17139

Our own experience is summarized in Table 1.

Patients with both the mild and severe forms of paralysis recover with astonishing rapidity in some cases, while in others the course of the disease is protracted and sometimes permanent paralysis results, as in the cases reported by Levy and Price.

Koch<sup>11</sup> believed that treatment paralysis was caused by street virus, modified by the treatment, and supported this claim by reporting the result of inoculation of a number of animals from the lumbar cord of a fatal case. Some of these animals died, after a long incubation period, with symptoms of rabies. This would indicate street virus infection.

When the cord method of preventive treatment is used, paralysis and neuritis are much more common after the intensive than after the milder forms of treatment. Many of the reported cases have occurred in cases with slight injuries. This would seem to us to indicate that the great majority of these cases are due to a fixed virus infection. Kozewalow<sup>12</sup> contended that fixed virus infection caused these symp-

<sup>1</sup> Jour. Am. Med. Assn., 1917, 69, p. 1873.

<sup>2</sup> Jour. Am. Med. Assn., 1916, 66, p. 1769.

<sup>3</sup> Semana Medica, Buenos Aires, 1917, 24, p. 1.

<sup>4</sup> Jour. Am. Med. Assn., 1917, 68, p. 513.

<sup>5</sup> Arch. de Med. Exper., 1916-17, 27, p. 387.

<sup>6</sup> Neurol. Centralbl., 1918, 37, p. 586.

<sup>7</sup> Deutsch. med. Wehnschr., 1916, 42, p. 874.

<sup>8</sup> Ztschr. f. Hyg. u. Infektionskr., 1918, 81, p. 85.

<sup>9</sup> Beitr. z. klin. Wehnschr., 1917-18, 6, p. 87.

<sup>10</sup> Jour. Nerv. & Ment. Dis., N. Y., 1917, 45, p. 67.

<sup>11</sup> Ztschr. f. Hyg. u. Infektionskr., 1909, 64, p. 258.

<sup>12</sup> Centralbl. f. Bakteriöl., O., 1, 1914, 73, p. 54.



toms, and seemingly proved his contention by citing one case of his own and seven other fatal cases in which portions of the cord and medulla, removed at necropsy and inoculated into rabbits, killed them in from 5 to 8 days, and showed that in incubation period and other characteristics the virus from these cases resembled fixed virus.

TABLE 1  
CASES OF TREATMENT PARALYSIS FOLLOWING ANTIRABIC INOCULATIONS

Cord Method. Schedule of Hygienic Laboratory.....1,680 cases								
Case	Sex	Age	Character of Injury	Animal	Diagnosis	Time of Onset	Character and Location	Duration and Results
1	M	30	Hands soiled by saliva	Dog	Laboratory	7 days after completion of treatment	Facial nerve, similar to Bell's palsy	Recovered in 8 days
2*	M	41	Lacerated arm	Dog	Laboratory	7 days after completion of treatment	Right hand and arm, similar to telegrapher's cramp	Recovered in 6 weeks
3	M	36	Hands soiled by saliva	Man	Clinical	8th day of treatment	Painful and tingling sensation on back of thumb and index and middle fingers; some loss of extension	Recovered in 3 mos.
4	F	40	5 punctures on leg and arm	Cat	Laboratory	9 days after completion of treatment	Patient apprehensive and depressed; tingling and very painful sensation on ear, side of face and neck	Almost well in 6 weeks
5	M	40	Lacerated arm	Dog	Laboratory	3 days after completion of treatment	Painful and tingling sensation with almost complete loss of function of entire injured arm; onset accompanied with nervous excitement and apprehension	Partial recovery when last seen 5 weeks after onset
6	F	29	Punctures, arm and forearm	Dog	Laboratory	10th day of treatment	Complete ascending paralysis extending above waist and involving sphincters	Recovery rapid, practically well in 4 weeks
Dilution method. (Approximately 1,000 minimal lethal doses for 13 to 18 days following 3 days dead virus).....2,268 cases treated								
7	M	34	2 punctures on wrist	Dog	Laboratory	8 days after completion of treatment	Severe pain in left side of abdomen and left leg which was partially paralyzed	In bed 1 week. Recovery practically complete in 3 months
Cumming Method. No cases out of.....59 cases treated								

As the symptomatology of these fatal cases, which have been investigated by inoculation of animals, did not differ from that of many severe cases of treatment paralysis that recovered, our conclusion is that at least some of the cases which recover are either infected with street virus of less virulence than usual, or with fixed virus, and in either event represent recoveries from rabies in man.

It should be noted that Hasseltine<sup>13</sup> and others contend that the paralysis is probably due to anaphylaxis from repeated injections of nerve tissue and cite the negative inoculation results of Babes'<sup>14</sup> case to uphold this contention, while others consider it a result of the injection of a hypothetical rabies toxin. These theories may explain certain of the cases, but not all of them, as it is difficult for us to believe that the brain of a person dying of anaphylaxis or from the effects of a toxin, should harbor living and virulent virus.

That treatment paralysis occurs also in animals is shown by a case reported to us by Dr. C. H. Case of Akron, Ohio.

A draft horse, bitten quite severely on the nose, was put under treatment on the fourth day after the injury. The treatment consisted of 3 days' injection of 75 mg. carbolized fixed virus, followed by 7 daily injections of approximately 10,000 minimal infective doses of living fixed virus.

On the 22nd day after the bite and the 18th day after the commencement of treatment the animal became nervous and excitable and refused to take the bit when attempts were made to harness him. He rapidly became weak and incoördinate in his gait, until scarcely able to move without falling. After 3 days' illness improvement began and was so rapid that in about a week the animal was put at light work.

This is the only case of trouble of any sort following antirabic treatment with massive doses of fixed virus, among 247 cases of horses, cows, and hogs, for which we have furnished treatment.

#### REPORTED RECOVERY IN MAN FROM NATURAL INFECTIONS

We can find no record of recovery in persons who have been infected with rabies by the bites of rabid animals and in whom the diagnosis has been confirmed by laboratory methods, but we wish to call attention to some of the reported recoveries in which the symptoms were so similar to those of hydrophobia that the attending physicians made this diagnosis.

In all the cases reported by Högyes<sup>15</sup> as possible recoveries in man, Pasteur treatment had been given so that treatment paralysis or fixed virus infection cannot be excluded.

In 1913, Moon<sup>16</sup> published a report on 3 inoculated dogs which recovered when bisulphate of quinin had been administered by mouth after the first symptoms had appeared.

<sup>13</sup> U. S. Pub. Health Service, 1913, p. 2220.

<sup>14</sup> Babes and Mironescu: *Compt. Rend. Soc. de Biol.*, 1908, 64, p. 964.

<sup>15</sup> Nothnagels *Spez. Path. u. Therap.*, 1897.

<sup>16</sup> *Jour. Infect. Dis.*, 1913, 13, p. 165

This stimulated the search for a specific drug and Harris<sup>17</sup> reported an apparent recovery, after the use of quinin, in a man who, after the Pasteur treatment was administered, developed symptoms of rabies, which would have been considered characteristic if he had died. Later experiments by Moon<sup>18</sup> failed to confirm the specific action of quinin reported in his earlier paper. Cumming,<sup>19</sup> Frothingham and Halliday<sup>20</sup> and Coward<sup>21</sup> using rabbits, and Krumweide and Mann<sup>22</sup> using rabbits and dogs, found that quinin did not prevent the appearance of rabies, and Fielder,<sup>23</sup> Geiger,<sup>24</sup> Wesson<sup>25</sup> and others failed to alter in the least the course of developed human rabies by quinin.

Considering the resistance of rabies virus to the action of phenol, one would not expect this chemical to have therapeutic value, but Haberman<sup>26</sup> reported an apparent cure of rabies in man by the use of phenol injected hypodermically. Fielder tried this method also, but his patient died.

An apparently "hopeless case" of rabies in a 13 year old child recovered when treated by R. Tonin<sup>27</sup> with 0.3 gm. of salvarsan together with KI, tepid baths, and stimulants. But the salvarsan treatment was ineffectual in human cases, when administered by Wesson and L'Arzt.<sup>28</sup> Mejio<sup>29</sup> found it of no benefit in treating man or animals with rabies and also showed that after death their brains were infectious.

With this evidence we must conclude that the specific cure for rabies has not been found. On the other hand, considering the great experience of some of the authors reporting apparent cures, it would seem possible that some of these cases might represent spontaneous cures, such as we herein report, as certainly occurring in one dog, and probably in a cow and in three other dogs.

It is to be hoped that all cases, not evidently hysterical, of persons recovering from an illness resembling rabies, and following the bite of an animal, be reported fully by clinicians, and that whenever possible inoculation into animals of the saliva of these persons should be made; if the person has not received Pasteur treatment his serum should be tested for rabicidal properties, after recovery. It should be remembered that the degree of virulence of the saliva of a rabid man has not been definitely determined, and, as is the case in other animals, is probably extremely variable. Much work remains to be done in regard to rabicidal serum.

<sup>17</sup> Jour. Am. Med. Assn., 1913, 61, p. 1511.

<sup>18</sup> Jour. Infect. Dis., 1915, 16, p. 58.

<sup>19</sup> Ibid., 1914, 15, p. 205.

<sup>20</sup> Jour. Med. Res., 1914, 30, p. 275.

<sup>21</sup> Southern Med. Jour., 1915, 8.

<sup>22</sup> Jour. Infect. Dis., 1915, 16, p. 24.

<sup>23</sup> Jour. Am. Med. Assn., 1916, 66, p. 1300.

<sup>24</sup> Calif. State Med. Jour., 1916, 14, p. 211.

<sup>25</sup> Jour. Am. Med. Assn., 1914, 62, p. 204.

<sup>26</sup> N. Y. State Jour. of Med., 1913, 13, p. 493.

<sup>27</sup> Policlinico, 1912.

<sup>28</sup> L'Art, Wien. klin. Wchnschr., 1917, 30, p. 1515.

<sup>29</sup> Semana Med., 1913, 20, p. 1301.

## RECOVERIES IN INOCULATED ANIMALS

Pasteur<sup>30</sup> was the first to establish the fact that recovery occasionally occurred in inoculated dogs after the first symptoms were manifest. Since that time Högyes reported that of 150 successfully inoculated dogs, 13 (8.1%) recovered after being ill with symptoms which he considered characteristic. Six of these had not been given the antirabic treatment.

Joseph Koch<sup>31</sup> reports three spontaneous recoveries of 40 experimentally infected dogs. These 3 had been inoculated with the same strain of street virus.

The case of recovery from rabies in a dog after a successful inoculation with fixed virus recorded by Damon and Hazencamp<sup>31</sup> is of especial interest since the diagnosis was confirmed by the reproduction of the disease in a rabbit inoculated with the saliva of the sick dog. A similar observation by Remlinger<sup>32</sup> is of even greater importance, because he not only confirmed the diagnosis by inoculation of guinea-pigs, while the dog was sick, but continued them after the dog's recovery and obtained positive results as late as 5 days after the dog was completely well. The diagnosis of rabies in the later pigs was confirmed by passage through rabbits.

Almost every worker in antirabic laboratories where fixed virus is standardized by inoculation into rabbits has, like Remlinger,<sup>33</sup> had the experience of seeing a rabbit inoculated with the higher dilutions, show symptoms of rabies and later recover. Koch states that recovery in rats and guinea-pigs has also been noted.

Heretofore it has been customary among physicians and veterinarians in this country to make a negative diagnosis of rabies whenever a man or animal showing rabiform symptoms recovered, but we do not think this course justified in the light of numerous articles which we have cited. Some of the published reports of poliomyelitis and similar conditions following the bite of a dog<sup>34</sup> will bear critical analysis.

## RECOVERIES IN ANIMALS AFTER NATURAL INFECTION

We have found reports similar to the first 3 herein recorded, in which there was a clinical history of natural rabies infection in animals with spontaneous recovery, but in which there was no confirmatory laboratory diagnosis. In our case 5 we consider the diagnosis established. In case 4 we consider the serologic findings suggestive.

CASE 1.—The following case was reported by Dr. Walter Brown of Columbus, Ohio, a veterinarian with a large clinical experience in canine diseases, who describes the case thus:

<sup>30</sup> Pasteur's Communication to the Academy, 1882, Expositions 9-10.

<sup>31</sup> Deutsch. tierarztl. Wehnschr., 1908, 16, p. 457.

<sup>32</sup> Compt. Rend. Soc. Biol., 1907, 62, p. 800.

<sup>33</sup> Ann. de l'Inst. Pasteur, 1919, 33, p. 735.

<sup>34</sup> Jour. Am. Med. Assn., 1912, 59, p. 2312.

"A large pointer dog suddenly became sullen and cross, and when seen was so aggressive that a close examination was impossible. He was secured, taken to the hospital, and placed in the rabies ward, where he refused to eat food, but ate his own feces, and seized any foreign bodies presented to him. He was very noisy and had the howl of a rabid dog.

"There was no history of exposure, but rabies was epizootic at that time. After about 3 days his hind quarters partially lost their coordination and his lower jaw showed the usual 'drop jaw' symptom so characteristic of dumb rabies. At the same time he became very weak, his gait was tottering, and his respirations noisy. He was no longer aggressive, but extremely restless, and looked like the ordinary case of paralytic rabies.

"His condition changed but little, excepting that he grew gradually weaker from day to day until about the eighth day, when he began to grow better; on the ninth day he drank a little and took some nourishment. From this time his improvement was steady, so that he was able to walk home, a considerable distance, in 2 weeks from his admission to the hospital. His jaws were still partially paralyzed.

"When some months later he had recovered full strength and use of his legs and body, excepting the jaws, which remained weak. A very considerable atrophy of the masseter muscles had occurred, from which the owner reports he never fully recovered."

CASE 2.—This report was made to us by Dr. J. V. Shoemaker, Department of Veterinary Medicine, Ohio State University. "The dog in question, a small fox terrier, was presented for examination Oct. 31, 1918. Eight weeks before, within 2 miles of the dog's home, a rabid dog had bitten 3 horses and a cat. One of the horses bitten was given Pasteur treatment and is now in good condition. The other 2 were not treated and both developed rabies about 5 weeks later. The owner of the patient reported that another terrier owned by him had disappeared 10 days before and was not heard of again.

"At the time of examination the patient had been sick one day and showed a vacant, staring expression, unequal pupils, paralysis of lower jaw and, in fact, all the symptoms which are typical of dumb rabies.

"No treatment was given, but the animal was kept under observation. His condition remained apparently unchanged until the 8th day, when improvement commenced. This continued gradually until Nov. 13, when he was discharged from the hospital and is alive and well at this date, 2 years later."

CASE 3.—This case was reported to us by Dr. Norton Dock, Cincinnati, Ohio. "A setter dog, 3 years of age, with no definite history of exposure, though rabies was prevalent in Cincinnati at the time, developed symptoms of dumb rabies,' such as general depression, paralysis of the lower jaw, dyspnea with its characteristic throat sounds. When he attempted to drink he was unable to swallow. His owner stated that the dog had been sick for several days. He was admitted to the hospital with a diagnosis of rabies. Two days later the dog seemed more animated and the angle between the mandibles was lessened. At this stage it was evident that his efforts to swallow were somewhat successful, both water and liquid food being taken after an effort. The dog rapidly became brighter and stronger so that he was taking solid food in a week's time, and its expression and appearance were practically normal. He was quarantined 14 days longer and dismissed. He was killed by a street car a year later."

CASE 4.—This case was reported to us by Dr. C. H. Case of Akron, Ohio. He was first called to see the cow July 4, 1920, and found her very gaunt and

TABLE 2  
EFFECT OF SERUM ON APPEARANCE OF FIRST SYMPTOMS AND COURSE OF DISEASE

Series 1, inoculated 9/9/20, 8 p. m., using fixed virus 328 B mixed with appropriate amount of serum to give dilutions indicated, and held for 30 min. at 37 C. Rabbits used for all tests.									
Series 1	Serum Dilution	9/15 A. M.	9/16 A. M.	9/17 A. M.	9/18 A. M.	9/19 A. M.	9/19 P. M.		
	Control (2)	1st Symp.	Sick	Died					
	Undiluted	.....	.....	1st Symp	Sick	Sick	Died		
	1:2	.....	1st Symp.	Sick	Died				
	1:5	.....	1st Symp.	Sick	Died				
	1:10	.....	.....	.....	.....	.....	.....	Lived	
	1:20	.....	.....	1st Symp.	Sick	Sick	Died		
	5 c c intraperitoneally (1)	.....	.....	1st Symp.	Sick	Sick	Died		
	1 c c intraperitoneally (1)	.....	.....	.....	.....	.....	.....	Lived	
Series 2, inoculated 9/18/20, 8:30 p. m. using fixed virus 325 A mixed with appropriate amount of serum to give dilutions indicated, and held for 24 hours at 20 C. Rabbits used for all tests.									
Series 2	Serum Dilution	9/24 A. M.	9/24 P. M.	9/25 A. M.	9/26 A. M.	9/26 P. M.	9/27 A. M.	9/27 P. M.	9/28 A. M.
	Control (2)	1st Symp.	Sick	Sick	Sick	Died			
	Control guinea-pig serum (3)	1st Symp. 1st Symp.	Sick Sick	Sick Sick	Sick Sick	Sick Sick	Sick Died	Died	
	Undiluted A B	1st Symp. 1st Symp.	Sick Sick	Sick Sick	Sick Died	Died Sick			
	1:5 A B	..... .....	..... .....	..... .....	Very slight symptoms 1st Symp.	Sick Sick	Sick Sick	Lived Died	
	1:10 A B	..... .....	..... .....	..... .....	..... .....	..... .....	..... .....	..... .....	Lived Lived
	1:20 A B	..... .....	..... .....	1st Symp. 1st Symp.	Sick Sick	Sick Sick	Sick Died	Died	
	1:50 A B	..... .....	1st Symp. 1st Symp.	Sick Sick	Sick Sick	Died Died			
	1:100 A B	1st Symp. 1st Symp.	Sick Sick	Sick Sick	Sick Sick	Died Sick	Died		
Series 3, inoculated 10/1/20, 8 p. m. using virus 323 A mixed with appropriate amount of serum to give dilution indicated, and held for 24 hours at 20 C. Guinea-pigs used for all tests.									
Series 3	Serum Dilution	10/5 A. M.	10/6 A. M.	10/7 A. M.	10/8 A. M.	10/9 A. M.			
	Control	.....	.....	1st Symp.	Died				
	1:10 A B	Died (traumatic)	.....	.....	.....	.....		Lived	
	1 c c intraperitoneally (1) A B	..... .....	..... .....	..... 1st Symp.	1st Symp. Sick	Died Died			

(1) The 2 rabbits in series 1 and 2, guinea-pigs in series 3 received untreated virus intracranially and undiluted serum intraperitoneally. In 2 other guinea-pigs injected similarly Sept. 20, 2.5 c c of serum were ineffective.

(2) For the controls in each series the virus was kept under the same conditions as the virus-serum mixture.

(3) In series 2, 0.5 c c of fresh normal guinea-pig serum was added as a control for any nonspecific serum effect on rabies virus.

not eating and frequently starting and running as though to escape from some terrifying object. The pupils were dilated, and she did not eat or drink but was salivated and drooled over her feed. She had given no milk that morning. Dr. Case instructed the owner to tie her securely in the barn and, avoiding contamination with the saliva, to await developments. The next day she showed the same symptoms but in addition had a peculiar bellow and attempted to attack persons passing before the stall. A diagnosis of rabies was made, and the owner was advised to keep her until she died so that he could claim reimbursement from the county for her loss.

Later it was learned from the owner that the condition remained unchanged for 3 days. Improvement then began and the cow drank and ate a little. In about 10 days the milk flow returned, and she was turned out to pasture. Rabies was prevalent in Akron all this year.

Astonished at such a recovery, Dr. Case reported it to us, and we determined to investigate it.

Dr. J. F. Planz took us to see the cow Sept. 7, and drew for us about 100 cc of blood from the jugular vein. At this time she appeared to be a normal, very placid cow.

The serum from the blood was tested for rabicidal properties (table 2). It will be noted in the 3 series of animals that dilutions of 1 part of serum to 9 parts of normal salt solution were found to have destroyed the infectivity of equal amounts of a 1% solution of fixed virus in salt solution. Slightly larger amounts of serum 0.2 cc to 0.8 cc salt solution (1 part in 5) prolonged the incubation approximately 48 hours, and 1 of 3 rabbits survived. Dilutions of 0.25 cc to 0.48 c (1 in 20) prolonged the incubation about the same length of time. Greater and less amounts of serum were without marked effect.

It is well known that rabicidal serum is often sharply limited in its action, and that the more intensive the immunization, the narrower the zones seem to be. We wish to call attention to the extremely limited zone of activity of this cow's serum, after apparent recovery from rabies.

CASE 5.—The animal, a cocker spaniel, was brought to the University Clinic June 3, 1918, by the owner, who stated that the dog had been bitten about 3 weeks previously by a neighbor's dog which had died soon after. The owner stated that the dog had acted as though choking and that he had kept his mouth partly open for the past 2 days.

The dog was much depressed, the lower jaw drooped, and the expression and voice were characteristic of rabies. He would not attempt to eat or drink. A clinical diagnosis of "dumb rabies" was made, and this was later confirmed by the entire clinical staff. The clinical diagnosis of rabies was further strengthened by the fact that 3 other dogs bitten on the head by the dog that had bitten this spaniel died of rabies after an incubation period of 13 to 15 days. During the 4 days following admission the condition became worse and rabies symptoms more marked. There was partial paralysis of the hind legs so that by the 4th day he was unable to stand. On the 5th day the condition was unchanged, but on the 6th a slight improvement was noticed, and he made efforts to eat. During the next 4 days improvement was plain and on the 10th day he could stand. Improvement continued fairly rapidly except the drooping jaw. After 15 days he could hold up his jaw for a time, but it continued to droop at intervals for 4 weeks. The general condition improved

greatly, and he was apparently normal on the 33d day after admission. He was killed on the 38th day. Inoculations of the brain into 2 rabbits intracranially and into guinea-pigs intramuscularly were negative, and no Negri bodies were found.

On the 9th day of the disease swabs, similar to those used for diagnostic purposes in diphtheria, were inserted in the dog's mouth and were allowed to rest near the opening of the submaxillary duct until saturated. These were then inserted into lacerated cuts in the nape of the neck of 2 young rabbits.

Rabbit A died 6 days later with general paralysis, following convulsions. Rabbit B died with typical symptoms of rabies 20 days after inoculation. The first of these rabbits proved particularly interesting as rabies virus was present in its brain even though the incubation was short and cultures from the brain showed contamination with gram-positive diplococci. By storing in glycerol at  $-2^{\circ}\text{C}$ . and inoculating into other animals, a sterile virus was obtained. This was carried through 5 passages as shown in table 3.

The only light which we can throw on the frequency of such recoveries is obtained by going over the records of the Veterinary Clinic, Ohio State University, for 9 years: there are 120 cases in dogs diagnosed as rabies with 2 recoveries, both of which are reported now.

No detailed clinical histories have been included in table 3, but all animals that died as a result of inoculation showed symptoms characteristic of inoculation rabies.

Cultures, not only from the brain but also from the viscera and heart blood, were made in dextrose broth fermentation tubes and on hormone-agar slants in each necropsy in which culture results are noted in the table.

Examinations for Negri bodies were made in smears prepared from Ammon's horn, the cerebral cortex, and the cerebellum. All smears were stained by Williams' method. Only in guinea-pigs 3 and 4 (fourth passage) were large intracellular Negri bodies found typical enough to make a definite positive diagnosis on these alone. In other instances either no bodies were found or, if present, they were so small and of such character that in our routine examination of rabies specimens we would have reported the case "very suspicious." It is worth noting in connection with our experience in demonstrating Negri bodies that Cruikshank and Wright<sup>35</sup> at the Pasteur Institute of Southern India, had quite similar results in their examination of the brains of many animals experimentally inoculated with the saliva of rabid animals. They conclude that "Negri bodies are not always demonstrable in the brains of experimental rabid animals, although they may become so after sub-passage."

<sup>35</sup> Indian Jour. Med. Res., 1914, 1, p. 562.



TABLE 3  
RESULTS OF INOCULATION TREATMENT FOR RABIES

Passage 1 Using Saliva of Clinically Rabid Dog, Which Recovered	Passage 2 Using Brain from Rabbit A	Passage 3 Using Brain of Rabbit A <sub>1</sub>	Passage 4 Using Brain from Rabbit A <sub>3</sub>	Passage 5 Using Brain from Dog 2
Rabbit A. June 12, 1918, inoculated in cut in nape of neck; died June 18, 1918; abscess at site of inoculation; cultures show gram-positive diplococci; smears from brain negative for Negri bodies	Rabbit A <sub>1</sub> . July 10, 1918, intracranial; died July 26, 1918; cultures from brain show gram-positive diplococci; smears from brain negative for Negri bodies  Rabbit A <sub>2</sub> . Dec. 2, 1918, intracranial; died Jan. 27, 1919; cultures negative; smears negative for Negri bodies	Rabbit A <sub>1</sub> . Dec. 2, 1918, intracranial; died Dec. 17, 1918; cultures negative; smears negative for Negri bodies  Using Brain of Rabbit 6  Rabbit 8. June 12, 1919, intracranial; developed stiffness and abscess of hind leg and was destroyed June 24, 1919  Guinea-pig 1. June 12, 1919, intracranial; died July 13, 1919; cultures negative; small extracellular Negri bodies found  Guinea-pig 2. June 12, 1919, intramuscular; died July 8, 1919; cultures negative; small extracellular; Negri bodies found	Dog 2. Feb. 10, 1919, intracranial; died Feb. 30, 1919; cultures negative; small extracellular bodies found  Dog 4. Feb. 28, 1919, intracranial; no symptoms; destroyed after 3 months  Dog 5. Feb. 28, 1919, intramuscular; no symptoms; destroyed after 3 months  Using Brain from Guinea-pig 1  Guinea-pig 3. July 13, 1919, intracranial; died Aug. 4, 1919; cultures negative; smears show typical intracellular Negri bodies  Guinea-pig 4. July 13, 1919, intracranial; died Aug. 6, 1919; cultures negative; smears show typical intracellular Negri bodies	Dog 3. Feb. 20, 1919, intracranial; died Feb. 27, 1919; cultures negative; smears negative for Negri bodies  (This animal developed rapid paralysis beginning February 26 and showed a tendency to snap at objects presented to him)

## CONCLUSIONS

Spontaneous recovery from rabies naturally acquired, while rare, does occur.

The saliva of an animal which recovers from rabies may have been extremely virulent during the course of the disease.

As early as 38 days after recovery from street rabies in a dog, the infectivity of the brain may disappear and Negri bodies be absent.

Therapeutic measures to control the symptoms in developed rabies in man should not be so heroic as to themselves endanger the life of the patient, for there is a possibility of recovery.

## THE INITIAL EXANTHEM OF SMALLPOX

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Prior to the peculiar eruption of smallpox is the so-called initial exanthem. This eruption bears a close resemblance to the eruptions of scarlet fever and measles, and is of significance in the diagnosis of smallpox, especially in cases of so-called variola sine exanthemate, when it may be the only symptom to base an intelligent diagnosis on.

Looking over the reports on the initial exanthem we find that they agree, in the main, but as it often does not appear, coming out more frequently in cases of varioloid, it may be passed over.

Both Hebra and Simon classified the initial exanthem into the two kinds, hemorrhagic and nonhemorrhagic. The hemorrhagic kind appears as a spotted erythema, resembling the eruption of scarlet fever. It is due to capillary hemorrhage, and the spots do not lose color under pressure. The nonhemorrhagic kind produces small spots of color, and although they look like measles they are not elevated from the skin. They vary in size from mere spots to lentils, and turn pale under digital pressure.

The hypogastrium, the inside of the thigh or Simon's thigh triangle, the side of the trunk to the axilla, the upper arm, the patella, etc., are regarded as the most likely parts to show the initial exanthem.

The initial rash lasts from 1 to 3 days. The nonhemorrhagic kind usually remains from 12 to 24 hours, and the hemorrhagic kind 3 or 4 days, until the appearance of the typical eruption.

The smallpox eruption proper fades in color, yet shows no scaling; on the parts where the initial eruption has appeared, smallpox eruption seldom comes forth.

From June, 1917, to May, 1918, 103 patients with smallpox were treated at the Dairen Isolation Hospital, and 39 patients had an initial exanthem. This figure should not be taken for anything like the ratio of the appearance of the initial exanthem, as there is reason to suppose that the actual number was larger, for the patients were admitted mostly after they had developed the characteristic pustules. In such cases, the initial exanthem had already disappeared, and the patients were asked

whether the initial exanthem had appeared and, if it had, as to its progress, but even in cases in which the answer was in the affirmative, it was difficult to ascertain if it really concerned the initial exanthem or the first stage of the smallpox eruption. Therefore, only such cases as were determined by us are given here.

*Form.*—In most cases the eruptions were of the size of the head of a pin, of a rosy color. In some cases there was erythema of irregular shape; the spots lost color under digital pressure, but at times there was capillary hemorrhage when the spots did not turn pale under pressure.

In a word, the shape closely resembles in some cases the scarlet fever eruption and in other cases the measles. For this reason, the initial exanthem is sometimes mistaken for scarlet fever, and the latter for the former, and the patients are hospitalized accordingly. However, on closer inspection it will be found that there are really three kinds, one like the scarlet fever eruption, one with hemorrhage, and one that resembles the measles rash.

Hebra and others say that the exanthem that resembles scarlet fever is attended with hemorrhage and does not turn pale under digital pressure. But, as a matter of fact, what resembles scarlet fever is not always attended with hemorrhage. On the contrary, cases accompanied with hemorrhage were few, there being only 4 cases in which the major portion of the eruption was hemorrhagic, and all were of a serious kind. Therefore, we are of the opinion that it is proper to classify these eruptions into 3 kinds. Yuhki, also, in his classification has made mention of the mixed type.

*Localization.*—The localization of the eruption is most characteristic, and often serves as guide to the diagnosis of the exanthem. The distribution of the initial exanthem is given in table 1.

The part in which the eruption is likeliest to appear is the outer side of the upper arm; next comes Simon's thigh triangle. Looking over the records available so far, no reference has been made to any close relation between the smallpox eruption and the outer side of the upper arm, except in four instances reported by Yuhki in Chugai Iji Shimpo, No. 718.

As a matter of fact, in almost every case of smallpox, eruption appears on the outer side of the upper arm. One noteworthy point is that a thicker eruption appears on the vaccinated side, even when the eruption appears all over the body, the eruption making its appearance first of all usually on the outer side of the upper arm, then spreading

TABLE 1  
DISTRIBUTION OF INITIAL EXANTHEM

No.	Sex	Age	Nature of Attack	Vaccination Marks		Distribution of Exanthem						Times Vaccinated	Time Since Last Vaccination
				Left	Right	Left Arm	Right Arm	Simon's Thigh Triangle	Abdomen	Chest	Back		
1	F	20	Light.....	6	5	+++	+	+	..	+	+	+	1 month, left
2	F	22	Light.....	4	5	+++	++	+	+	+	+	+	1 month, left
3	M	40	Medium.....	0	0	+++	++	+	+	+	+	+	5 yrs., left and right
4	F	22	Light.....	0	2	+++	+	+	+	+	+	+	7 months, left
5	F	30	Serious.....	3	3	+++	++	+	+	+	+	+	10 years
6	F	24	Medium.....	3	4	+++	++	+	+	+	+	+	5 yrs., left and right
7	F	40	Serious.....	3	3	+++	++	+	+	+	+	+	1 yr., left and right
8	F	26	Light.....	2	4	+++	++	+	+	+	+	+	1 year, left
9	M	44	Death.....	0	0	+++	++	+	+	+	+	+	10 years
10	M	32	Medium.....	3	2	+++	++	+	+	+	+	+	7 mo., left and right
11	M	39	Light.....	3	1	+++	++	+	+	+	+	+	3 years
12	F	22	Light.....	2	2	+++	++	+	+	+	+	+	1 year, left
13	M	41	Death.....	8	6	+++	++	+	+	+	+	+	2 yrs., left and right
14	F	25	Light.....	3	2	+++	++	+	+	+	+	+	7 months, left
15	M	34	Serious.....	5	3	+++	++	+	+	+	+	+	10 years
16	F	17	Light.....	4	3	+++	++	+	+	+	+	+	6 months, left
17	F	24	Death.....	1	1	+++	++	+	+	+	+	+	10 years
18	F	54	Light.....	0	0	+++	++	+	+	+	+	+	1 year, left
19	M	14	Light.....	5	2	+++	++	+	+	+	+	+	1 yr., left
20	M	46	Serious.....	3	2	+++	++	+	+	+	+	+	3 mo., left and right
21	M	36	Serious.....	4	4	+++	++	+	+	+	+	+	1 yr., left and right
22	F	21	Light.....	3	2	+++	++	+	+	+	+	+	16 days, left
23	F	25	Light.....	0	0	+++	++	+	+	+	+	+	11 days, left
24	F	25	Light.....	2	2	+++	+	+	+	+	+	+	22 days, left
25	F	22	Light.....	0	0	+++	+	+	+	+	+	+	29 days, left
26	M	22	Death.....	0	0	+++	++	+	+	+	+	+	8 years
27	M	28	Light.....	0	4	+++	++	+	+	+	+	+	3 months, left
28	F	37	Light.....	1	0	+++	++	+	+	+	+	+	30 days, left
29	F	43	Light.....	2	2	+++	++	+	+	+	+	+	9 days, left
30	M	47	Medium.....	4	3	+++	++	+	+	+	+	+	2 yrs., left and right
31	F	36	Medium.....	3	3	+++	++	+	+	+	+	+	1 yr., left and right
32	M	25	Light.....	2	4	+++	++	+	+	+	+	+	2 years, right
33	M	24	Medium.....	3	6	+++	++	+	+	+	+	+	2 months, left
34	M	36	Light.....	6	0	+++	++	+	+	+	+	+	2 years, left
35	F	30	Medium.....	4	1	+++	++	+	+	+	+	+	1 year, left
36	F	25	Light.....	2	5	+++	++	+	+	+	+	+	10 days, left
37	M	23	Light.....	2	5	+++	++	+	+	+	+	+	2 yrs., left
38	F	26	Medium.....	2	0	+++	++	+	+	+	+	+	7 days, left
39	F	26	Light.....	0	0	+++	++	+	+	+	+	+	7 days, left
Totals.....				39	34	27	19	22	16	21	21	21	
Degrees in density of exanthem.....				13	4	5	19	22	16	21	21	21	
				14	16	22	19	22	16	21	21	21	
				12	4	22	19	22	16	21	21	21	

to other parts. Even when the eruption appears simultaneously on both sides or all over the body, it is remarkable that the part vaccinated not long before shows a thicker eruption than elsewhere.

As regards the cause of the initial exanthem, it is not yet known, but it may be inferred that there exists some relation between the localization of the eruption and vaccination. In 2 nonvaccinated babies, although the eruption appeared all over the body, that on the outer side of the upper arm was not any thicker than elsewhere. In order to put the point to test we picked out 3 young white rabbits and shaved the hair off on both sides of the back, vaccinating each at 3 points on one of the sides. We waited about a fortnight until the local inflammation had faded, and then mixing vaccine lymph with salt solution at a certain ratio, a small quantity of the mixture was inoculated underneath the skin. It was noticed that in one of the 3 rabbits the vaccinated part became flushed.

To sum up: It seems a peculiar phenomenon in those that have been vaccinated once that the initial exanthem appears on the outer side of the upper arm. On this account, in case of doubt as to the nature of an eruption looking like scarlet fever or measles rash, if the localization is limited to the outer side of the upper arm, or the eruption makes its first appearance here, or is particularly thick here, the case may with reasonable certainty be regarded as one of smallpox.

*Relation to Pock Marks.*—From the table we note that in 7 of the 39 cases the eruption appeared conspicuously in parts on which there were few pock marks. The presence of many vaccination marks on one side did not prevent the same side from showing comparatively few eruptions. On the other hand, the eruption came out thick on the other side. There were two such instances in our cases. Hence the number of vaccination marks and the extent of the initial exanthem do not necessarily coincide, indicating that there is no direct relation between the eruption and vaccination marks.

*Relation to the Last Vaccination.*—Supposing that while the initial exanthem bears some relation to vaccination it has no direct relation to smallpox marks, we must take note of the time that elapsed after the last vaccination, in other words, the relation between the last vaccination and the initial exanthem must be studied.

The table shows that in almost every case the part newly vaccinated showed a greater density of eruption than elsewhere, or was the spot of the first appearance of the eruption, followed by eruption on the other side.

However, in cases in which ten years had elapsed after the last vaccination, this relation could not be ascertained; indeed, it is indicated that there is no relation. Among the cases under our treatment there were only 3 or 4 such instances, hence no conclusion may be drawn. However, considering the fact that the eruption was remarkably thick on the vaccinated side (especially when the vaccination was recent), it might be said that the initial exanthem is apt to break out in the vaccinated part for some time, this tendency decreasing as time passes until the vaccinated part reacts as do other parts.

*Relation Between Nature of Disease and Prognosis.*—The initial exanthem appears oftenest in cases of varioloid, and cases with initial exanthem generally give light symptoms. Of the 39 cases under our treatment, 9 were serious, 8 less serious, and 22 light. Still it must not be concluded hastily that all the cases showing initial exanthems are light ones, as especially those attended with hemorrhage had an extremely unfavorable prognosis, 4 of 9 in the table terminating fatally. Even among the less serious cases there were some in which the characteristic eruption thickly covered all the body, taking a good length of time for recovery. In other words, the appearance of the initial exanthem cannot always be taken as a reliable guide to prognosis.

#### CONCLUSIONS

Close observation seems to warrant the classification of the initial exanthem in smallpox into the hemorrhagic, the scarlet-fever-like, and the measles-like.

The eruption appeared on the outer side of the upper arm in all the cases in this series.

There is no direct relation between the initial exanthem and the pock marks.

In the vaccinated the eruption is most marked about the place of vaccination. However, if ten years have elapsed since the last vaccination, no such tendency is apparent.

An initial exanthem does not necessarily guarantee a light attack. This is true especially if the exanthem is hemorrhagic.

## B. BOTULINUS (TYPE A) ASSOCIATED WITH FATAL PASTURE DISEASE OF HORSES

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For several years the writers have been studying, as the opportunity has been presented, the phenomena of an acute and subacute syndrome of horses grazing in pasture. The range and topography of the districts from which the outbreaks have been reported have suggested that the virus of the disease is rather widespread in nature or possibly indigenous to certain localities, though clarification of the epizootologic facts, as well as the development of specific diagnostic and prophylactic measures, obviously await a definite knowledge of the causative factor or factors involved. If the conjecture relative to the widespread character of the virus is correct, it seems apparent that the sporadic appearance of the disease in pasture-fed horses is closely dependent on certain unknown natural influences which seasonally retard or facilitate the development of the disease and entrance to the host.

Since there is no assurance of the specificity of the remotely occurring outbreaks, it seems advisable in a preliminary survey to mention the fact that the feed has been suspected, though it has not been definitely incriminated. Pearson,<sup>1</sup> Milks,<sup>2</sup> Hickman,<sup>3</sup> Mohler,<sup>4</sup> Uhdall,<sup>5</sup> Haslem,<sup>6</sup> Stange<sup>7</sup> and others have recognized the potentiality of a food poisoning in equine outbreaks of an epizootic and sporadic nature, though the agreement or disagreement regarding the relation of feed to the disease has been a matter of opinion rather than judgment based on specifically controlled experimental data. The clinical symptoms enumerated by different investigators are somewhat analogous, and at present constitute the only possible evidence of a common etiology.

A disease of horses occurring on pasture, which clinically resembles equine botulism, has been reported in several states, including eastern, southern and middle western localities. Inability to communicate or

Received for publication, March 11, 1921.

<sup>1</sup> Report of State Veterinarian, Department of Agriculture, Pennsylvania, 1902.

<sup>2</sup> Louisiana Exper. Station Bull. 106, 1908.

<sup>3</sup> Bureau of Animal Industry, Department of Agriculture, Circular 122, 1906.

<sup>4</sup> Bureau of Animal Industry, Department of Agriculture, Bull. 65, 1914.

<sup>5</sup> Cornell Veterinarian, 1913.

<sup>6</sup> Kansas Exper. Station Bull. 173, 1910.

<sup>7</sup> American Veterinary Review, 42, 1913.



B. BOTULINUS A IN PASTURE DISEASE OF HORSES 115

to transmit artificially the syndrome by blood inoculation or by association, together with negative bacteriologic findings in cultivating the aerobic flora of the visceral organs and heart's blood of fatally afflicted animals, has in a large measure stifled progress in the etiologic studies of the disease. In fact, the recorded results of laboratory investigations have repeatedly failed to explain the character of the losses in question, though negative findings in recent years have invited attention to the long neglected study of the anaerobes associated with certain spontaneous outbreaks of this or similar diseases.

Different phases of this investigation are still in progress, but since some time may elapse before a complete resumé will seem desirable, the authors are submitting a brief report on a portion of the bacteriologic work wherein attention has been given to the presence of spore-bearing anaerobes. This report is based on the occurrence of a fatal endemic disease in horses which was brought to our attention through specimens presented for examination by Dr. J. H. McNeil, Chief of the Bureau of Animal Industry, Trenton, N. J.

HISTORY OF SPECIMENS

An endemic disease of horses of varying severity has occurred in certain pasture districts in New Jersey for many years. The extent of the losses has been variable in different seasons, but the recurrence of the disease has attracted the attention of state sanitary officials. The majority of the severe outbreaks have been noted during the late summer and fall months, though sporadic cases have probably occurred throughout the year. The symptoms in affected horses, as observed by Dr. McNeil during the months of August, September, October, and November, 1920, suggested to him the possibility of a botulinus intoxication, though up to that time no evidence, to the knowledge of the writers, had been obtained to show that botulism occurred among grazing animals. The incidence of this disease in pasture-fed horses implied that botulinus toxin had developed in uncut grass or forage or that water supplies had been contaminated.

B. BOTULINUS A SYMBIOTIC AEROBE

In certain fatal, obscure, and toxemic-like diseases in domestic animals, sometimes referred to as forage poisoning, bacteriologic studies suggest that *B. botulinus*, type A or B, may have been a primary factor. The sporadic losses in question involved cattle, sheep, and swine

throughout certain localities of the Mississippi Valley. In the same vicinities type A botulinus toxin was apparently a primary factor in the development of so-called "limber neck" of the domestic fowl. In these outbreaks harvested grain and forage, including ensilage, bakery refuse and restaurant garbage, and even certain mineral tonics prepared for animals, were found contaminated with *B. botulinus*. Each of these feeds apparently provided anaerobic conditions for the development of the toxin. *B. botulinus* has also been found in the intestinal contents and spleen and brain tissue of fatally afflicted animals, although evidence of growth in vivo with elaboration of toxin has not, in our studies, been observed.

The fact that *B. botulinus* is an anaerobe might lead to the belief that botulinus toxin would be excluded per se in pasture-fed animals. It does not necessarily follow, however, that anaerobiosis is essential to the development of the toxin. Growth of *B. botulinus* occurs aerobically in vitro in association with *B. subtilis* unprotected from light. Symbiotic development is also accompanied by the characteristic toxin production. Furthermore, it has been observed that the botulinus toxin in grain and hay is water diffusible, suggesting the possibility of the water supplies in improperly drained pasture lands becoming contaminated. In fact, certain outbreaks of a fatal disease in horses with manifest symptoms of botulism have been observed by veterinarians wherein the uncut grass and water of undrained fields were tentatively suspected. The relation of the water supplies to the disease in question should be intensively studied, although in the outbreak under discussion no attempt has been made by the writers to incriminate the water. It is apparent from the results, however, that either the water or the grass or perhaps both, might be involved under certain conditions. The investigation of the outbreak in New Jersey has been restricted to the study of a strain of *B. botulinus* (type A) encountered in the spleen of typically afflicted animals following death.

#### B. BOTULINUS (TYPE A) ISOLATED FROM SPLEEN

Several specimens, including spleen, brain and intestinal contents of fatally afflicted horses, together with a sample of hay from the pasture in which the horses had died, were subjected to bacteriologic study. One spleen specimen (539) proved to be contaminated with a

gram-positive spore-bearing anaerobe, 0.9-1 by 3-8 mikrons, indistinguishable from *B. botulinus*, type A. The culturally sterile toxin produced by this strain in amounts of 0.00001 c c is fatal in less than 24 hours to guinea-pigs weighing 250 gm. Antitoxin prepared from a

TABLE 1  
IMMUNOLOGIC RELATION OF STRAINS 84 AND 539

Weight of Guinea-Pigs in Gm.	Treatment Given at 10 a. m., Oct. 26, 1920		Results
	Antitoxin	Toxin 539	
300	25 units A* (No. 84)	0.3 c c†	Remained healthy
300	25 units B (No. 126)	0.3 c c	Dead, 8 a. m., Oct. 27, 1920
300	.....	0.3 c c	Dead, 5 p. m., Oct. 26, 1920

\* Antitoxin given subcutaneously

† Toxin given per os.

strain of *B. botulinus*, type A (84), isolated from olives, proved efficacious in protecting guinea-pigs against a lethal amount of toxin (539), as shown in table 1.

Antitoxin prepared from *B. botulinus* type A (539) apparently affords specific protection to guinea-pigs receiving fatal amounts of

TABLE 2  
IMMUNOLOGIC RELATION OF STRAINS 539 AND 1500

Weight of Guinea-Pig in Gm.	Treatment Given at 9 a. m., Jan. 28, 1921		Results
	Antitoxin	Toxin	
300	25 units A* (No. 539)	0.1 c c spinach liquor† (No. 1500)	Remained healthy
300	25 units B (No. 126)	0.1 c c spinach liquor (No. 1500)	Dead, 8 p. m., Jan. 29, 1921
300	.....	0.1 c c spinach liquor (No. 1500)	Dead, 8 p. m., Jan. 28, 1921

\* Antitoxin given subcutaneously

† Toxin given per os.

toxin from remotely isolated type A strains. The results of typing tests to determine the specificity of the antitoxin prepared from *B. botulinus* toxin (539) against type A toxins present in the liquor of spinach (samples 1500 and 1592, associated with fatal outbreaks of botulism in man) are shown in tables 2 and 3.

## B. BOTULINUS NOT FOUND IN HAY

Repeated bacteriologic examinations of a sample of hay from one of the suspected New Jersey pastures failed to reveal the presence of *B. botulinus* or other toxic anaerobes, though negative evidence in one sample cannot be considered conclusive regarding the relation of the hay or grass to the disease in question. The fact that the rations of the afflicted horses were limited to pasture grass implies that the toxin was probably present either in the uncut hay or in the water consumed by the affected animals. A toxic anaerobe was encountered in one speci-

TABLE 3  
IMMUNOLOGIC RELATION OF STRAINS 539 AND 1592

Weight of Guinea-Pigs in Gm.	Treatment Given at 9 a. m., Feb. 15, 1921		Results
	Antitoxin	Toxin	
275	25 units A* (No. 539)	0.1 c c spinach liquor† (No. 1592)	Remained healthy
275	25 units B (No. 126)	0.1 c c spinach liquor (No. 1592)	Dead, 8 a. m., Feb. 16, 1921
275	.....	0.1 c c spinach liquor (No. 1592)	Dead, 8 a. m., Feb. 16, 1921

\* Antitoxin given subcutaneously

† Toxin given per os.

men of intestinal contents, but in attempting to purify the strain and eliminate nonpathogenic types it was lost. Cultures from the brain of one specimen submitted proved negative to *B. botulinus*.

The bacteriologic and immunologic results of this investigation suggest to the authors that the losses experienced in certain New Jersey pasture-fed horses may be traceable to *B. botulinus* type A, though the relation, if any, of this intoxication to a similar enzootic disease of horses in remote outbreaks in other parts of America remains to be established.

## SUMMARY

*B. botulinus* type A (539) was isolated from the spleen of a horse that displayed typical symptoms of an endemic syndrome occurring in certain pasture-fed horses in New Jersey.

In immunologic tests, as well as cultural examinations, this strain is indistinguishable from *B. botulinus* occurring in olives and spinach which proved to be the cause of outbreaks of botulism in man.

On repeated examination the one sample of hay obtained from a pasture in which the horses had died proved free from *B. botulinus* and other toxic anaerobes.

Until the pathogenic and saprophytic characters of *B. botulinus* throughout its cycle in nature are more definitely established, the significance of the type A strain found in the equine spleen is not definitely concluded. In the light of our present knowledge, fortified by clinical evidence in afflicted animals, the bacteriologic findings herein recorded may be diagnostic of equine botulism.

# AN INVESTIGATION INTO THE PURITY OF AMERICAN STRAINS OF BACILLUS BOTULINUS

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The descriptions of *Bacillus botulinus* by the various investigators of this country differ markedly in certain respects from those of van Ermengem<sup>1</sup> and other European investigators. What is particularly noteworthy is that odors of putrefactive decomposition were not noted by the latter.

In a recent study of *B. botulinus* the author employed 19 different strains of the organism. These were obtained from various laboratories and came originally from many sections of the United States. A preliminary examination of some of these strains soon gave some indications of impurities in the cultures. Consequently, all of the 19 strains were subjected to anaerobic plate cultivation. In every instance but one toxic and nontoxic cultures were obtained from the stock strains. In the one exceptional case only a single attempt was made. Nontoxic cultures were obtained from some of the stock strains only after repeated efforts. These had all the characteristics of *B. sporogenes*.

The method of isolation of *B. botulinus* quite generally employed by American investigators may be of particular significance here. Inoculations were made into deep glucose-agar tubes, and after certain periods of incubation transfers were made from colonies in the depths of the agar. It is true that on some occasions preliminary enrichment and dilution were resorted to, but in few, if any, instances were the transfers made from exposed surface colonies. Graham and Brueckner<sup>2</sup> made their transfers from deep colonies developing in glucose gelatin plates.

McIntosh and Fildes<sup>3</sup> criticize the deep agar method of isolation of anaerobes as tending to yield impure cultures. They further state: "It cannot here be impressed too strongly on the worker that the purity

Received for publication, March 20, 1921.

<sup>1</sup> Centralbl. f. Bakteriöl., O., 1896, 19, p. 442; Ztschr. f. Hyg. u. Infektionskr., 1897, 26, p. 1.

<sup>2</sup> J. Bacteriol., 1919, 4, p. 1.

<sup>3</sup> Med. Research Com., Special Report Series 12, 1917.

of anaerobes can only be tested and controlled by repeated surface cultivation." Van Ermengem and other European investigators succeeded in their isolation of *B. botulinus* by the use of the anaerobic plating method.

The author's method of preparing anaerobic plates, which differs little from that of Stoddard,<sup>4</sup> is as follows:

From the supernatant liquid of a 2 to 4 day culture of *B. botulinus* in egg-meat medium 0.2 to 0.3 cc is added to 10 cc of sterile salt solution in a test tube containing well-washed sand. The tube is closed with a previously sterilized rubber stopper and shaken vigorously for 3 or 4 minutes; 0.5 cc of this suspension is added to a second 10 cc of salt solution and sand and shaken as before. This process is again repeated. With a bent glass rod the final suspension is streaked over the surface of glucose-agar plates which have been dried at 37 C. over night to eliminate excessive moisture from the agar surface. These plates are incubated for 3 days at 34 C. in a closed jar from which the oxygen has been removed by the hydrogen replacement and vacuum pump exhaustion method.

By this method an organism in every way resembling *B. sporogenes* was isolated from all but one of the stock cultures. Isolations of *B. sporogenes* were made from original "strains" of *B. botulinus* by four different methods, namely, by the procedure described in the foregoing, by simple dilution, by aerobic plating with *Staphylococcus aureus* (Sturges), and by the heating of young cultures which contained only the spores of *B. sporogenes* and vegetative forms of *B. botulinus*.

The isolations of the nontoxic strain (*B. sporogenes*) were made from colonies closely resembling those of *B. sporogenes*. The non-toxicity was determined by the injection of 1 cc of a 1:10 dilution of the supernatant fluid of egg-meat cultures into the peritoneal cavity of the white mouse. All the cultures which proved non-toxic were further incubated for 3 weeks at room temperature, and again tested for toxicity. Furthermore, tests were also carried out on guinea-pigs. After 4 days' feeding of the guinea-pigs with these strains grown for 4 weeks in egg-meat medium no symptoms of poisoning were observed. On the other hand, a small amount of material from toxic cultures obtained from the same plates and prepared in the same way killed the guinea-pig in less than 24 hours.

Toxic cultures were obtained from colonies which were more or less smooth and regular; and again from others which were less smooth or which even resembled typical colonies of *B. sporogenes*. All of

<sup>4</sup> Jour. Am. Med. Assn., 1918, 70, p. 906.

these toxic cultures decomposed the egg-meat medium with the evolution of foul odor, but less extensively and less rapidly than did the nontoxic cultures. Their toxicity was determined in the same way as those which proved by animal experimentation to be innocuous. It appears from the evidence at hand that none of these toxic stock "strains" should be considered as pure cultures. It is quite apparent, however, that they contain the *B. botulinus*, whereas the nontoxic strains were freed from this organism, and in every way resembled pure cultures of *B. sporogenes*.

A comparative study was made of the toxic and nontoxic "strains" in their relation to gas production from glucose, lactose, levulose, galactose, maltose, and sucrose, with these results: The nontoxic strains readily attacked glucose, levulose and maltose, whereas the toxic "strains," while exerting the same action on the glucose, levulose and

TABLE 1  
SHOWING REPRESENTATIVE GAS PRODUCTION IN THE SUGAR BROTHS BY THE TOXIC AND NONTOXIC CULTURES

Cultures	Dextrose Percentage	Lactose	Sucrose	Maltose Percentage	Levulose Percentage	Galactose
Nontoxic.....	80	0	0	40	15	Occasional bubble
Toxic.....	80	+	+	40	15	Occasional bubble

maltose as did the nontoxic, also produced a small amount of gas from lactose and sucrose. An occasional bubble of gas was produced in galactose broth by both. Table 1 serves to illustrate the differences in gas-producing power in the different sugar mediums.

Inability to attack lactose and sucrose is characteristic of *B. sporogenes*. The action on glucose, maltose and levulose is also typical of this anaerobe. Furthermore, the morphology and various cultural characteristics, particularly the rapid decomposition of egg-meat, with the production of offensive odors, are added evidence that the nontoxic strains isolated from the original toxic stock cultures are *B. sporogenes*. The occasional formation of gas in the galactose broth tubes may be attributed to slight impurity in the galactose, though all of the carbohydrates employed were of the highest purity obtainable. The basic beef-extract broth was shown to be free from dextrose by inoculation with *B. coli* and a stock strain of *B. sporogenes*. The test mediums were made from plain beef-extract broth ( $P_H$  7.2 to 8.0), to which 1% of



the carbohydrate was added. The medium was filled into large double-barreled Durham fermentation tubes in liberal amount, and covered with a layer of high grade neutral mineral oil.

In table 2 are given the sugar reactions and toxicity tests of two representative nontoxic strains isolated from each so-called "strain" of *B. botulinus* tested; also the reactions of the toxic isolations from the strain from which nontoxic cultures were not obtained. The organisms included in the first part of the table were isolated from sporogenes-like colonies and were similar in their action on meat mediums and milk to pure cultures of *B. sporogenes*; the others were obtained by 3 other methods. They are all gram-positive anaerobes, with subterminal spores. Toxicity tests were made on white mice, a 1:10 dilution of the supernatant liquid of a 7 to 10 day old egg-meat culture at 37 C. being injected intraperitoneally. If symptoms of botulism and death did not occur within 48 hours, the inoculation test was considered as negative and the culture nontoxic. The sub-strains from strain 8 of this series, although showing *B. sporogenes*-like colonies, were all toxic. This strain was plated only once. The toxicity of the organisms fished from *B. sporogenes*-like colonies may be explained on the assumption that *B. botulinus* spores were within the colony, but that they had not germinated, or that little multiplication had taken place. It is quite probable that if further attempts had been made to isolate nontoxic substrains from this stock strain, such would have been obtained. The plating technic was checked in every possible way to make sure that no contamination had been introduced. The original "strains" were tested occasionally to determine their constancy. Nontoxic strains were isolated from one culture by the simple dilution method which Sturges and Rettger<sup>5</sup> used with such success in isolating *B. putrificus*. A microscopic count was made of the rods and spores in a salt suspension of one of the stock strains of *B. botulinus*, and this diluted so that 1 c.c. contained about 2 organisms spores or rods.

One c.c. of this dilution was inoculated into each of six egg-meat tubes. Dilutions made to contain 1 bacillus or spore per c.c. and one-half per c.c., respectively, were also inoculated into egg-meat tubes and incubated at 37 C. for 10 days. At the end of this time the tubes showing growth were tested for toxicity. Four of the tubes diluted to contain approximately two organisms per c.c. showed growth; one from each of the other series showed the same kind of growth. These were

<sup>5</sup> Jour. Bacteriol., 1919, 4, p. 171.

found to be nontoxic by mouse inoculation. The action on egg-meat medium was like that of *B. sporogenes*, being more rapid than the control (the original strain inoculated at the same time). The sugar reactions also were those of *B. sporogenes*, as is shown in table 2 (see BI and BII). *B. sporogenes* was evidently present in numbers far

TABLE 2  
SHOWING SUGAR REACTIONS (Gas) AND TOXICITY OF *B. SPOROGENES*-LIKE STRAINS ISOLATED FROM THE ORIGINAL STOCK "STRAINS" OF *B. BOTULINUS*

Laboratory Number of Culture	Percent Gas Formed in						Toxicity Tests on White Mice
	Dex- trose	Lac- tose	Suc- rose	Mal- tose	Levu- lose	Galac- tose	
Isolated by the anaerobic plate method:							
1 (2).....	90	0	0	60	10	B	—
1 (3).....	95	0	0	60	20	B	—
2 (1).....	80	0	0	50	15	0	—
2 (4).....	100	0	0	20	+	0	—
3 (2).....	100	0	0	90	10	0	—
3 (5).....	60	0	0	70	15	0	—
4 (1).....	50	0	0	70	60	0	—
4 (2).....	70	0	0	80	60	0	—
5 (1).....	95	0	0	40	10	0	—
5 (6).....	70	0	0	50	10	0	—
6 (2).....	50	0	0	60	50	B	—
6 (3).....	70	B	0	50	40	B	—
7 (1).....	70	0	0	60	30	0	—
7 (2).....	60	0	0	40	40	B	—
8 (2).....	50	10	+	60	70	B	—
8 (3).....	60	+	+	30	60	B	—
9 (1).....	90	0	0	30	10	0	—
9 (7).....	80	0	0	30	10	0	—
10 (3).....	75	0	0	20	10	B	—
10 (4).....	60	0	0	30	10	B	—
11 (1).....	75	0	0	90	+	0	—
11 (2).....	60	0	0	80	10	0	—
12 (2).....	60	0	0	40	10	0	—
12 (8).....	60	0	0	30	10	B	—
13 (2).....	60	0	0	30	10	0	—
13 (5).....	100	0	0	40	+	0	—
14 (4).....	60	0	0	100	10	0	—
14 (5).....	50	0	0	30	10	0	—
17 (2).....	50	0	0	60	+	0	—
17 (6).....	50	0	0	50	+	0	—
18 (1).....	80	0	0	60	10	0	—
18 (3).....	40	0	0	60	10	B	—
19 (1).....	80	0	0	30	10	0	—
19 (9).....	50	0	0	30	10	0	—
A (1).....	40	0	0	60	10	0	—
A (5).....	80	0	0	90	15	B	—
Isolated by the simple dilution method							
B I.....	100	0	0	90	15	0	—
B II.....	90	0	0	70	30	B	—
Isolated by the aerobic method of Sturges, involving the use of <i>Staphylococcus aureus</i> :							
3+St.....	70	0	0	40	20	B	—
4+St.....	60	0	0	50	20	0	—
Isolated by the heating method:							
B 20.....	80	0	0	50	15	B	—

superior to the numbers of *B. botulinus*. This is probably true in many cases, as shown by 2 other strains from which *B. sporogenes* was isolated by the dilution method with the aid of *Staphylococcus aureus* (see 3 + St. and 4 + St. in table 2).

Following the other method recommended by Sturges and Rettger, a suspension made up of a mixture of one of the stock strains of *B. botulinus* and of *Staphylococcus aureus* was plated aerobically, dilutions containing different proportions of these two organisms being plated in series. These were incubated for 2 days at 37 C. Fifteen colonies were fished from plates in each series, inoculated into egg-meat medium and incubated at 37 C. for 10 days. Only 3 out of 45 inoculated tubes showed anaerobic growth, the others containing *Staphylococcus aureus* only. One series of plates was made in the same way from another stock strain of *B. botulinus* and *Staphylococcus aureus* and from this only one isolation was effected which contained an anaerobe, all the other cultures being pure *Staphylococcus aureus*. All 4 of the anaerobic cultures proved to be nontoxic and, after *Staphylococcus aureus* had been eliminated by heating at 80 C. for 15 minutes, gave all the characteristic reactions of *B. sporogenes*. The nontoxicity of these cultures was confirmed later after a prolonged incubation at room temperature.

These results indicate that *B. sporogenes* is present in predominating numbers in at least some strains of *B. botulinus*.

*B. botulinus* has been described by van Ermengem and other European bacteriologists as being a slow spore former. In a special report the British Medical Research Committee in 1919 describes *B. botulinus* as not forming spores readily, and maintains that these spores when formed are small and do not distend the rod, a character quite different from that ascribed to the organism isolated by American investigators. Spores have been observed in the strains of the series here investigated after an incubation at 37 C. of only one or two days. These spores distended the ends of the rods to the same degree as is shown in *B. sporogenes* cultures grown in the same medium. To determine whether or not the spores were those of *B. botulinus* or *B. sporogenes* the following experiment was performed: four egg-meat tubes and 4 dextrose broth tubes (with a layer of oil on the broth) were inoculated with one of the stock strains of *B. botulinus*, and all incubated at 37 C. At intervals of 12 hours one inoculated tube of each medium was heated at 80-85 C. for 20 minutes. At the end of 48 hours, when all of the tubes had been subjected to heat for the time required to kill vegetative forms, all were inoculated into fresh tubes of egg-meat and incubated at 37 C. for 17 days. The results are given in table 3, sugar reactions included.

It is apparent from table 3 that the *B. botulinus* spores did not form until some time between the thirty-sixth and forty-eighth hour after inoculation, but it is also shown that some spores were formed and that the cultures from these surviving spores were nontoxic. The meat medium was digested with the production of putrefactive odor, and the meat was somewhat darkened. The egg-meat medium recommended by Rettger<sup>6</sup> was used to show proteolytic property. At the end of the first 12 hours not all of the spores of *B. botulinus* in the meat medium had germinated, hence the toxic culture obtained after heating at 80 C. McIntosh and Fildes state that *B. sporogenes* spores are formed readily and are quite numerous even in the first 21 hours of incubation. This was proved in the foregoing experiment. The non-toxicity of those cultures indicated as nontoxic in the table was confirmed after longer incubation, by feeding to a guinea-pig.

TABLE 3  
RESULTS OF EXPERIMENT WITH SPORES

Stock Strain	Number Hours Incubated	Medium	Percentage Gas in						Toxicity
			Dextrose	Lactose	Sucrose	Maltose	Levulose	Galactose	
"B"	12	Meat	50	0	+	40	20	Bubble	+
"B"	12	Broth	50	0	0	60	15	0	—
"B"	24	Meat	60	0	0	60	15	0	—
"B"	24	Broth	80	0	0	50	15	Bubble	—
"B"	36	Meat	40	0	0	70	30	Bubble	—
"B"	36	Broth	40	0	0	60	10	Bubble	—
"B"	48	Meat	50	+	+	60	10	Bubble	+
"B"	48	Broth	30	+	+	60	20	Bubble	+

The finding of *B. sporogenes* in *B. botulinus* cultures is not a new experience. Burke, in a personal communication, indicates that *B. sporogenes* is liable to be present in such cultures and describes the appearance in broth of such contaminated cultures. One of the bacteriologic laboratories of this country found the American Museum of Natural History strains of *B. botulinus* to be, in reality, *B. sporogenes*. The contaminant in this instance presumably had entirely suppressed and supplanted the toxic *B. botulinus*. Among the strains first obtained by Shippen<sup>7</sup> when he began his studies on *B. botulinus*, some were found to be nontoxic. These were strains received by Dickson<sup>8</sup> from Zinsser and the American Museum of Natural History. Nevin had also noted that these strains had lost their toxic property. It now

<sup>6</sup> Jour. Biol. Chem., 1906, 2, p. 71.

<sup>7</sup> Arch. Int. Med., 1919, 23, p. 346.

<sup>8</sup> Rockefeller Inst. for Med. Research, Monograph 8, Botulism, 1918.

appears that what actually happened was that the nontoxic *B. sporogenes*, which was present in the cultures as a contaminant, supplanted the toxic *B. botulinus*. The presence of this rapidly-growing anaerobe in the American strains of *B. botulinus* probably accounts for the varying degrees of toxicity which are accorded them in the literature. This will explain, too, the apparent loss of toxicity of certain American strains.

*B. sporogenes* is admittedly one of the most widely disseminated of anaerobes. It is common in the classes of materials from which the American strains of *B. botulinus* have been isolated. It is well known, furthermore, that *B. sporogenes* grows luxuriantly with other organisms. When *B. sporogenes* and *B. botulinus* are present in the same material they are so closely associated that it becomes a difficult task to separate them. *B. sporogenes*, being the less fastidious of the two, is favored by the method of isolation used by American workers. By heating the infected material before diluting in agar, the more resistant form, *B. sporogenes*, not only survives, but gains the ascendancy over *B. botulinus*. McIntosh recognized this fact and hence did not kill nonspore forms by heating, in his isolation of spore-bearing anaerobes from pus, etc.

The so-called American strains of *B. botulinus* have been obtained from materials which were, beyond all doubt, contaminated with *B. sporogenes*. Such materials were in most cases in an active state of decomposition or spoilage. Dickson<sup>8</sup> reports the origin of the strains which he isolated up to the beginning of 1917, and refers to the food from which the isolations were made as being spoiled. Burke<sup>9</sup> isolated strains of *B. botulinus* from sources which are ordinarily heavily seeded with *B. sporogenes*, for example, moldy hay, manure from a hog which had recovered from botulism, scarred bush beans, spiders from a bush bean plant, etc. *B. botulinus* in such materials would probably be closely associated with *B. sporogenes*, from which it would be extremely difficult to separate it. Graham and Brueckner<sup>2</sup> obtained their strains from moldy hay soiled with chicken excreta, and from silage, materials which in all probability were contaminated with *B. sporogenes*. Thom, Edmondson and Giltner<sup>10</sup> isolated *B. botulinus* from canned asparagus which showed perceptible signs of spoilage. Armstrong<sup>11</sup> obtained his organism from olives which had caused a small epidemic of botulism

<sup>8</sup> Jour. Bact., 1919, 4, p. 541.

<sup>10</sup> Jour. Am. Med. Assn., 1919, 73, p. 907.

<sup>11</sup> Public Health Reports, 1919, 34, p. 2877.

and which "smelled like limberger cheese." Edmondson, DeBord and Thom,<sup>12</sup> reporting results of bacteriologic tests of 510 samples of canned olives, found *B. botulinus* in 7 cans, all of which showed signs of spoilage. Emerson and Collins,<sup>13</sup> on the other hand, isolated their strain from canned olives which gave an odor resembling that of rancid butter.

Most European strains have been isolated from sources relatively free from this contaminant. Putrefactive decomposition is not associated with the occurrence of the organism in the food from which they were obtained. Van Ermengem<sup>1</sup> procured the original strain of *B. botulinus* from an apparently normal ham which showed no signs of decomposition or spoilage. The organism isolated did not give repulsive odors in any medium used, even in meat, peptone bouillon, etc. Only a sour odor is mentioned, the absence of any foul odors being emphasized. Römer<sup>14</sup> obtained his strain of *B. botulinus* from a portion of a ham which had been exposed to the air and which had lost its natural color and consistency, had turned greenish, and which had developed a butyric acid, but not putrefactive odor. No mention was made of putrefactive decomposition. Madsen<sup>15</sup> isolated *B. botulinus* from fish which had an intense butyric odor and which had caused botulism. Van Ermengem in 1906 again obtained a strain similar to the one previously isolated, from another ham which had a disagreeable, more or less rancid, odor, but he does not mention any signs of putrefactive decomposition. In one place on the ham there was a brownish spot which was of a slimy consistency; from this area the isolation was made. Landmann,<sup>16</sup> in reporting his work on the Darmstadt outbreak, did not mention any spoilage of the beans which had caused the outbreak. Schumacher<sup>17</sup> described the ham which was responsible for 6 cases of botulism as smelling rancid. Ornstein<sup>18</sup> reported the deaths of 2 persons who had eaten ham which had merely a butyric odor. These descriptions of the materials from which *B. botulinus* was isolated by European bacteriologists are in more or less contrast to those recorded by American investigators. The absence of foul odors accounts, in part, for the apparent ease with which the European strains were

<sup>12</sup> Bacteriol. Abstracts, 1919, 4, p. 10.

<sup>13</sup> Jour. Lab. & Clin. Med., 1920, 5, p. 559.

<sup>14</sup> Centralbl. f. Bakteriöl., 1900, 27, p. 857.

<sup>15</sup> Cited by Van Ermengem, Kolle and Wassermann, Handbuch, 1912, 4, p. 909.

<sup>16</sup> Hyg. Rundschau, 1904, 14, p. 449.

<sup>17</sup> Münch. med. Wehnschr., 1913, 60, p. 124.

<sup>18</sup> Ztschr. f. Chemotherapie, 1912, 1, p. 458.

obtained pure, for *B. sporogenes* was probably not present in large numbers, if at all.

No mention is made by the European investigators of proteolytic properties of their strains other than gelatin liquefaction. On the other hand, the American strains have been described as being decidedly proteolytic, digesting meat with the production of the characteristic odors of putrefaction and peptonizing milk. Gelatin is liquefied, of course. The van Ermengem strain does not grow well in milk and does not coagulate or peptonize it (van Ermengem<sup>15</sup>). McIntosh and Fildes state that it does not grow well in meat either. This is in marked contrast to the reported cultural characters of the American strains. Von Hibler,<sup>19</sup> however, working with a strain of *B. botulinus* obtained from the Kral (Vienna) collection, describes it as being decidedly proteolytic, digesting coagulated serum and clotting and peptonizing milk. McIntosh and Fildes<sup>3</sup> found *B. sporogenes* in the strain of *B. botulinus* obtained by them from Kral, the source of von Hibler's strains. Van Ermengem claims von Hibler was working with cultures which were of very low toxicity. The presence of *B. sporogenes* in the culture could easily account for the low toxicity if it were present in predominating numbers. Dickson compared his isolations with the strains of *B. botulinus* described by von Hibler and noted the same proteolytic activity in milk as von Hibler did.

The American strains obtained by the writer from various sources are indistinguishable from each other culturally, including the Dickson strains. They all digest meat with the evolution of putrefactive gases. They peptonize milk in the same manner as pure cultures of *B. sporogenes*, but in some cases not quite so rapidly. In meat the action of *B. sporogenes* is more rapid than that of the *B. botulinus* stock strains, but the appearance of the medium is quite similar. At first the odor from the latter is not so strongly putrefactive as later, slightly resembling that of butyric acid during the first day's incubation.

It cannot be said that any of the toxic "strains" are pure *B. botulinus*. They, like the original stock cultures, show every indication of being mixed cultures of *B. botulinus* and *B. sporogenes*. The surface colonies which answered the description of typical colonies of *B. botulinus* contained, besides the botulism organism, at least a small number of *B. sporogenes*. On the other hand, some of the typical *B. sporogenes* colonies were proved to enclose *B. botulinus* bacilli or spores.

<sup>19</sup> Untersuchungen über die anaeroben, 1908.

Inability of the author thus far to isolate *B. botulinus* may be explained readily as follows: This organism is slow to develop from single isolated cells, hence fails to appear in pure colony form. *B. sporogenes* is a hardy anaerobe, and may be isolated without difficulty from almost any source. On the agar plates this organism favors the former. Other examples of organisms which are isolated only with the greatest difficulty are *B. putrificus*, and perhaps to a somewhat lesser extent, *B. tetani*. It can be no exaggeration to say that very few cultures of *B. putrificus* have as yet been obtained, and that among the stock cultures of *B. tetani* in the different laboratories few are pure cultures.

The sources from which American strains of *B. botulinus* have been obtained have been such that close association of this organism with *B. sporogenes* must be taken as a matter of fact. Therefore, the problem of isolation of the botulism bacillus is naturally a difficult one. Attempts now being made by the author to obtain pure cultures which answer the description, or at least proximate, that given by van Ermengem and other European investigators, include long and repeated enrichment cultivation in particularly favorable environment, that is, special carbohydrate mediums, etc.

The finding of *B. sporogenes* as a contaminant of the American strains of *B. botulinus* is of considerable importance. The results explain clearly the reasons for the sharp differences noted in the descriptions of the organism given by the European and American investigators. The use of impure cultures in the making of antiserum is unsatisfactory and may account for the absence of uniformity of results in the treatment of botulism victims. The use of antisera in the prophylaxis of botulism has given good results experimentally in some instances, but it has not as yet been pronounced a success in actual practice. The toxicity of the strains of *B. botulinus* which are contaminated with *B. sporogenes* will vary with the age of the culture and with the proportion in which the contaminant is present.

The differences noted between the so-called "strains" of the present collection and the descriptions of *B. botulinus* given by van Ermengem and other European bacteriologists are so marked as to exclude them from being members of the same species. The marked proteolytic property alone of the American "strains" is sufficient to separate them entirely from the European strains. These differences can readily be accounted for by the presence of a contaminant like *B. sporogenes* in the American "strains."



## SUMMARY

Nontoxic strains were isolated from 18 out of 19 so-called "strains" of *B. botulinus* examined. These nontoxic isolations were proved to be *B. sporogenes*.

Isolations of *B. sporogenes* were made from stock "strains" of *B. botulinus* by 4 different methods, that is, by the use of anaerobic plates, simple dilution, aerobic plates with *Staphylococcus aureus*, and by heating in order to kill vegetative forms after the spores of *B. sporogenes* had been formed.

The "strains" of *B. botulinus* examined resemble morphologically and in their action on meat mediums and milk pure cultures of *B. sporogenes*, except that the action of *B. sporogenes* is more rapid, and in some cases carried nearer to completion.

# AN IMMUNOLOGIC STUDY OF BACILLUS INFLUENZAE

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This communication deals with a portion of a joint investigation to determine if any evidence of an immunologic nature could be adduced which would point toward an etiologic relationship between *Bacillus influenzae* of Pfeiffer and epidemic influenza.

The problem was approached from three standpoints; first, the search for agglutinins for *B. influenzae* in the serum of convalescents from epidemic influenza; second, the search for complement-fixing bodies in the same material; and third, the study of the immunologic relationships of the various strains of *B. influenzae* obtained from patients with epidemic influenza. Papers dealing with these three phases of the problem have already appeared.<sup>1</sup> It is the purpose now to record the results of a study of the immunologic relationship of strains of *B. influenzae* obtained from adults during the course of an attack of epidemic influenza.

The importance of this phase of the problem has been repeatedly emphasized by Park and was clearly in mind at the time these studies were instituted. To quote from Park<sup>2</sup>: "With the coming of the 1918 pandemic we knew that if the influenza bacillus was the initiating cause, we must be dealing with a single type of exalted virulence or toxicity or both, and that unless we could recover influenza bacilli from the large majority of early cases and show a definite antibody relationship between these strains, we could not add evidence as to its being the exciting factor." The occurrence of influenza in epidemic form in the early months of 1920 gave an opportunity for work along these lines, and for testing the results of previous investigators in this field.

## HISTORICAL

A review of all the bacteriologic work carried out on cases of influenza during the recent pandemic is considered beyond the scope of

Received for publication March 31, 1921.

<sup>1</sup> Utheim, Kirsten: *Jour. Infect. Dis.*, 1920, 27, p. 460; Cooke, J. V.: *Ibid.*, 476; Bell, H. H.: *Ibid.*, 464.

<sup>2</sup> Park, W. H., and Williams, A. H.: *Amer. Jour. Pub. Health*, 1919, 9, p. 45.

this paper. I shall confine myself to a summary of the studies that have been made in connection with the immunologic relationship of strains of influenza bacilli recovered from cases of epidemic influenza.

Gay and Harris<sup>3</sup> noted that a polyvalent serum produced by immunizing with several strains of *B. influenzae* failed to agglutinate two strains of this organism that had not been used for immunization, and suggested the existence of separate groups of *B. influenzae*. Huntoon and Hannum<sup>4</sup> and Roos<sup>5</sup> found evidence of immunologic relationship in the strains they studied. Fleming and Clemenger<sup>6</sup> tested 8 strains and found cross agglutination in one instance only. The most extensive researches in this problem have been those carried out by Valentine and Cooper<sup>7</sup> working under Park's direction. In a study of 181 strains from cases of influenza they found identical strains in different patients in 2 instances only and no evidence of immunologic grouping. On the other hand, Small and Dickson,<sup>8</sup> from a study of 10 strains, concluded that they could distinguish 4 groups on the basis of agglutination and absorption tests. Bell,<sup>1</sup> whose contribution forms part of the joint investigation mentioned, concluded from a study of 36 strains that "the influenza bacillus represents a heterogeneous group of organisms as shown by agglutination and absorption tests" and that identical strains do occur. Furthermore, he was unable to differentiate by those methods between organisms isolated from normal throats during an interval of 2 months prior to the recurrent epidemic of 1920, and those isolated from the throats of influenza patients during that epidemic. More recently, Povitzky and Denny<sup>9</sup> found 4 out of 7 strains obtained from cases of influenzal meningitis and isolated years apart which proved to belong to one immunologic group, and from respiratory cases they found as many as 5 strains from different individuals which belonged to the same group, although they were not able to find any other groups consisting of more than 2 members each. Coca and Kelley,<sup>10</sup> in a study of 18 strains, "isolated in different localities and at different times" found "identities in the cultures only when a probability of personal contact existed."

Apparently no one as yet has succeeded in recovering from cases of epidemic influenza a large proportion of strains of influenza bacilli that are identical in their immunologic relationships.

#### SOURCE OF MATERIAL

This work deals with 12 strains of influenza bacilli obtained from adults with influenza who were patients in the Barnes Hospital on the service of Dr. Dock. They were obtained during the course of the epidemic of influenza which prevailed in St. Louis during January and February, 1920. They were obtained by nasopharyngeal culture, by

<sup>3</sup> Jour. Infect. Dis., 1919, 25, p. 414.

<sup>4</sup> Jour Immunol., 1919, 4, p. 167.

<sup>5</sup> Jour. Immunol., *ibid.*, p. 189.

<sup>6</sup> Lancet, 1919, 2, p. 869.

<sup>7</sup> Jour. Immunol., 1919, 4, p. 359.

<sup>8</sup> Jour. Infect. Dis., 1920, 26, p. 230.

<sup>9</sup> Jour. Immunol., 1921, 6, p. 65.

<sup>10</sup> *Ibid.*, p. 87.

direct culture of washed sputum or by inoculation of sputum into white mice. They were considered to be influenza bacilli if they were small, pleomorphic bacilli, gram-negative, nonmotile, producing pin-point colonies on blood agar and demanding hemoglobin for their growth. They were isolated originally on 5% rabbit-blood agar and were kept growing on "chocolate agar."

#### TECHNIC

Rabbits were immunized with suspensions of living influenza bacilli in physiologic salt solution, washed from the surface of "chocolate-agar" plates. Intravenous injections were made at intervals of from 4 to 7 days until preliminary agglutination tests showed an agglutinin titer in the rabbit serum of 1:800 or more for the homologous strain. The animals were then bled and the serum stored in the refrigerator without preservative until used. The animals stood immunization fairly well, only one being lost, and it died shortly after the first injection.

Cross agglutination tests were carried out at 56 C. for 18 hours, preliminary experiments by Dr. Bell having shown that agglutination was sharper when carried out in this manner than if carried out at a lower temperature or for a shorter period of time. The highest dilution at which agglutination was just visible with the naked eye was the one recorded.

Considerable difficulty was encountered in getting some of the strains to remain in suspension. This factor of poor emulsibility seemed to be a constant characteristic of these particular strains, appearing almost uniformly, and was only overcome by a brief preliminary centrifugation of the suspension, thus throwing down the larger clumps and leaving a supernatant fluid which represented a thin suspension of the organisms in question. By this technic spontaneous agglutination was avoided with all the strains but one. This particular strain was so persistent in its spontaneous agglutination that it had to be discarded.

Absorption experiments were carried out at 56 C. for 4 hours, followed by 18 hours' exposure in the refrigerator. If, after this degree of exposure, preliminary tests still showed the presence in the serum of agglutinins for the absorbing strain, the procedure was repeated until all of the agglutinins for that strain were absorbed. For these absorption experiments a heavy suspension of the organisms was added to equal parts of whole serum.

#### RESULTS

Cross agglutination tests were carried out with all 12 strains and their homologous serums in the manner outlined, and the results are

recorded in table 2. In recording the titer the ultimate dilution is stated. A consideration of this table shows that strains 2, 3, 4 and 8 showed cross agglutination in dilutions ranging from 1:1200 to 1:3200. Strain 8 was less readily agglutinated than the other three strains. With the exception of these strains, no other evidence of cross agglutination in high dilution occurred. The serums of these 4 strains did not always agglutinate other strains to a like extent. For example, serum 2, serum 4, and serum 8 agglutinated strain 5 in a dilution of 1:50 whereas serum 3 agglutinated this strain in a dilution of 1:100. Nor were these strains (2, 3, 4 and 8) that gave evidence of cross agglutination always agglutinated to the same degree by heterologous serum. For example, serum 1 agglutinated strains 2 and 3 in a dilution of 1:50, but did not agglutinate strains 4 or 8. Other examples could be cited.

TABLE 1  
DIRECT AGGLUTINATION OF STRAINS OF INFLUENZA BACILLI WITH HOMOLOGOUS  
IMMUNE SERUMS  
Agglutination at 56 C. for 18 hours

Strains	Normal Rabbit Serum	Immune Serums											
		1	2	3	4	5	6	7	8	9	10	11	12
1	0	800	25	50	0	25	0	0	0	0	100	25	25
2	0	50	3,200	3,200	1,600	50	100	100	1,200	100	50	50	25
3	0	50	3,200	3,200	1,600	50	100	100	1,200	100	100	50	25
4	0	0	3,200	3,200	1,600	50	200	200	1,200	0	100	50	50
5	0	0	50	100	50	1,600	200	0	50	0	50	100	100
6	0	50	25	200	50	50	800	400	100	0	50	100	50
7	0	100	25	100	100	50	200	2,400	0	0	100	25	25
8	0	0	1,600	1,200	1,200	50	100	1,600	50	0	50	25	25
9	0	0	50	100	50	50	200	100	50	1,600	0	100	50
10	0	50	100	100	50	50	0	200	0	0	1,200	100	100
11	0	0	25	200	0	100	200	0	100	0	100	1,600	200
12	0	50	50	50	0	25	200	50	0	0	200	200	800

Among the 12 strains there were 5 (2, 3, 5, 11 and 12) whose serums agglutinated all heterologous strains in varying dilutions, but of these 5 there were only 2 (2 and 3) whose serum agglutinated other strains in high dilution, the limit of agglutination of the other four serums for heterologous strains being 1:200.

Except for strains 2, 3, 4 and 8, no other strains gave evidence of falling into a single immunologic group, as determined by the phenomenon of agglutination. These 4 strains, however, as they showed cross agglutination in dilutions ranging from 1:1200 to 1:3200, one would be inclined to regard as closely related if not identical from an immunologic point of view. To determine the correctness of this view resort was had to absorption experiments. For the absorption experiments the 4 strains giving cross agglutination reactions and 3 other

TABLE 2  
CROSS AGGLUTINATION EXPERIMENTS AFTER ABSORPTION

Serum for Strain 2			Serum for Strain 3			Serum for Strain 4			Serum for Strain 8		
Ab- sorbed with Strains	Tit- rated with Strains	Titer after Absorp- tion	Ab- sorbed with Strains	Tit- rated with Strains	Titer after Absorp- tion	Ab- sorbed with Strains	Tit- rated with Strains	Titer after Absorp- tion	Ab- sorbed with Strains	Tit- rated with Strains	Titer after Absorp- tion
2	2	0	2	2	0	2	2	0	2	2	0
2	3	0	2	3	25	2	3	0	2	3	25
2	4	0	2	4	0	2	4	0	2	4	0
2	5	0	2	5	25	2	5	0	2	5	0
2	6	0	2	6	25	2	6	0	2	6	0
2	8	0	2	8	0	2	8	0	2	8	25
2	9	0	2	9	25	2	9	0	2	9	0
3	2	0	3	2	0	3	2	0	3	2	0
3	3	0	3	3	0	3	3	0	3	3	0
3	4	0	3	4	0	3	4	0	3	4	0
3	5	0	3	5	0	3	5	0	3	5	0
3	6	0	3	6	0	3	6	0	3	6	0
3	8	0	3	8	0	3	8	0	3	8	25
3	9	0	3	9	0	3	9	0	3	9	0
4	2	0	4	2	25	4	2	0	4	2	0
4	3	0	4	3	25	4	3	0	4	3	0
4	4	0	4	4	0	4	4	0	4	4	0
4	5	0	4	5	25	4	5	0	4	5	0
4	6	0	4	6	25	4	6	0	4	6	0
4	8	0	4	8	0	4	8	0	4	8	25
4	9	0	4	9	25	4	9	0	4	9	0
5	2	3,200	5	2	1,600	5	2	1,600	5	2	800
5	3	1,600	5	3	1,600	5	3	1,600	5	3	800
5	4	3,200	5	4	1,600	5	4	1,600	5	4	800
5	5	0	5	5	0	5	5	0	5	5	0
5	6	25	5	6	25	5	6	50	5	6	50
5	8	800	5	8	800	5	8	1,200	5	8	800
5	9	50	5	9	50	5	9	50	5	9	25
6	2	1,600	6	2	3,200	6	2	1,600	6	2	800
6	3	1,600	6	3	3,200	6	3	1,600	6	3	800
6	4	1,600	6	4	3,200	6	4	1,600	6	4	800
6	5	50	6	5	25	6	5	50	6	5	25
6	6	0	6	6	0	6	6	0	6	6	0
6	8	800	6	8	1,200	6	8	1,200	6	8	1,200
6	9	25	6	9	50	6	9	25	6	9	25
8	2	0	8	2	0	8	2	0	8	2	0
8	3	0	8	3	0	8	3	0	8	3	0
8	4	0	8	4	0	8	4	0	8	4	0
8	5	0	8	5	0	8	5	0	8	5	0
8	6	0	8	6	0	8	6	0	8	6	0
8	8	0	8	8	0	8	8	0	8	8	0
8	9	0	8	9	0	8	9	0	8	9	0
9	2	800	9	2	3,200	9	2	800	9	2	800
9	3	800	9	3	3,200	9	3	800	9	3	800
9	4	800	9	4	3,200	9	4	800	9	4	800
9	5	25	9	5	100	9	5	50	9	5	25
9	6	25	9	6	200	9	6	25	9	6	0
9	8	1,600	9	8	1,200	9	8	400	9	8	1,200
9	9	0	9	9	0	9	9	0	9	9	0
Unab- sorbed	2	3,200	Unab- sorbed	2	3,200	Unab- sorbed	2	1,600	Unab- sorbed	2	800
Unab- sorbed	3	3,200	Unab- sorbed	3	3,200	Unab- sorbed	3	1,600	Unab- sorbed	3	800
Con- trol	4	3,200	Con- trol	4	3,200	Con- trol	4	1,600	Con- trol	4	800
Con- trol	5	50	Con- trol	5	100	Con- trol	5	50	Con- trol	5	25
Con- trol	6	25	Con- trol	6	200	Con- trol	6	50	Con- trol	6	50
Con- trol	8	1,600	Con- trol	8	1,200	Con- trol	8	1,200	Con- trol	8	1,200
Con- trol	9	50	Con- trol	9	100	Con- trol	9	50	Con- trol	9	25

strains (5, 6 and 9) were selected. Absorption of serums 2, 3, 4 and 8 was carried out with these 7 strains as described, and the selected strains were then agglutinated with the absorbed serums. The results of these experiments are shown in table 2.

From a study of this table it is seen that when serum 2 was absorbed with the homologous strain, not only were the agglutinins for that strain removed, but likewise the agglutinins for all the other strains tested, and when it was absorbed with strains 3, 4 and 8, which agglutinated with it in high dilution, likewise all agglutinins for the other strains were removed. When, however, the serum was absorbed with strains 5, 6 and 9, which were not originally agglutinated by it in high

TABLE 3

DIRECT AGGLUTINATION OF STRAINS OF BACILLUS INFLUENZAE WITH ANTI-B. INFLUENZAE SERUM OBTAINED FROM DR. BELL

Agglutination at 56 C. for 18 hours

Strains	Serum												
	B. Ous- lander	Burds- ley	Dock	Cot- ton	198	Bell	Fergu- son	Shinde- wolf	Par- sons	Ger- hart	Esse- mann	Bun- yard	Laser- sohn
1	0	0	25	0	0	0	0	0	0	0	0	0	0
2	25	50	100	200	100	0	0	0	400	25	0	0	200
3	0	50	100	200	100	0	0	0	400	25	0	0	200
4	0	50	100	50	100	0	0	0	200	50	0	0	100
5	0	0	200	200	0	0	0	0	0	0	0	0	0
6	0	0	100	0	0	0	0	0	0	0	25	0	0
8	0	0	50	400	0	0	0	0	25	0	0	0	0
9	0	0	50	0	200	0	0	0	25	0	50	0	0
10	0	25	200	50	0	100	50	0	0	100	0	50	25
11	0	0	200	0	0	0	200	0	0	0	0	0	0
12	0	0	400	0	0	0	0	0	0	0	0	0	0
Homologous strain	1,200	1,600	3,200	1,200	800	1,600	1,600	3,200	3,200	3,200	1,600	3,200	1,600

dilution, the specific agglutinins for the absorbing organism were removed, whereas those for the other organisms were either not removed or were reduced by an insignificant amount.

Analogous results were encountered when serum from strains 3, 4 and 8 was absorbed, except that in the case of serum 3 and serum 8 the absorbed serum still agglutinated other strains in a dilution of 1:25 as contrasted with dilutions of from 1:1200 to 1:3200 previous to absorption. Cross agglutination after absorption, therefore, bore out previous agglutination results and indicated that in the case of strains 2, 3, 4 and 8 we were dealing with strains that were identical from an immunologic standpoint.

It seems evident, therefore, from these experiments that among the 12 strains studied 4 were encountered which were capable of being

placed in one common immunologic group on the basis of agglutination and absorption tests, and that the remaining 8 gave no evidence which would justify placing them in immunologic groups.

Opportunity was afforded, through the kindness of Dr. Bell, to carry out a series of agglutinations with serum obtained by immunizing rabbits with strains of influenza bacilli. Thirteen such specimens were studied, and the results are shown in table 3. This table shows that 13 specimens of anti-serum derived from another source failed to agglutinate in high dilution any of the strains originally isolated by us, thus indicating that the strains used by Dr. Bell for obtaining these serums were not closely related to ours from an immunologic standpoint.

#### DISCUSSION

If *B. influenzae* of Pfeiffer is the etiologic agent of epidemic influenza, then, according to Park,<sup>2</sup> in view of the rapidity of the spread of this disease, we must assume that we are dealing with a strain of this organism which has a very high invasive power for man. That the "virus" of influenza may increase in invasive power within a short space of time is entirely conceivable. In a report<sup>11</sup> on an epidemic of influenza occurring in an isolated post in the American Expeditionary Forces we found evidence in support of this view. In this particular epidemic successive bodies of troops hitherto free from influenza were exposed to the disease and the incidence of the disease was greater in each succeeding body of troops, even when precautions had been taken to destroy any virus that might have been left behind by the preceding group of men.

If, however, a strain or several strains of *B. influenzae* acquire increased virulence to the extent that they may cause large proportions of the exposed population to come down with the disease in a short period of time, it is scarcely conceivable that such strains would not show close immunologic relationship. As Park has emphasized, we should expect to find in any group of patients during the acute stage of influenza a large number of strains of the influenza bacillus showing identity from an immunologic standpoint. It is true that the epidemic-producing strain might be missed, but the work of Valentine and Cooper,<sup>7</sup> and of Povitzky and Denny<sup>9</sup> showed that strains of this organism isolated from a single patient were almost uniformly identical in their antigenic behavior.

<sup>11</sup> Chesney, A. M., and Snow, F. W.: *Jour. Lab. & Clin. Med.*, 1920, 6, p. 78.



This study of 12 strains isolated from patients during the recurrent epidemic of 1920 demonstrates that 4, or 33⅓% were identical, and the remaining 8 gave no evidence of close immunologic relationship. When the study was completed, it was found by reference to the records of the laboratory that 2 of them, Nos. 3 and 4, had been isolated from the same patient, one (3) by nasopharyngeal culture, the other (4) by inoculation of sputum into mice. This fact was not known at the time the experiments were being carried out. When it is taken into consideration, however, the percentage of identical strains encountered in 11 different patients drops to 27.

We are inclined to consider that this percentage is too small to be of any real significance from the standpoint of etiological relationship of the disease. Although it is not beyond the realms of conjecture that these identical strains which we isolated are representatives of a strain responsible for the epidemic, we are far from claiming that such is the case. We think that a far greater percentage of identical strains should be obtained before concluding that this organism bears a causal relationship to epidemic influenza. We are therefore in substantial agreement with the view of those who hold that in the case of influenza bacilli we are dealing with a group of heterogeneous organisms, some of which may be related immunologically, but that the majority of them, even when obtained from influenza patients, are not capable of being placed in one immunologic group.

It was the intention, before reporting this work, to study these strains from the standpoint of their biochemical reactions as has been done by Jordan,<sup>12</sup> and Stillman and Bourn,<sup>13</sup> but through an unfortunate accident 9 of the strains were lost, and this fact necessitated the abandonment of the work at this point.

#### SUMMARY AND CONCLUSIONS

Twelve strains of hemoglobinophilic bacilli obtained from 11 patients with influenza during the recurrent epidemic of 1920 have been studied immunologically.

Cross-agglutination tests and absorption experiments indicate that of the 12 strains 4, or 33⅓% were identical in their immunologic reactions.

No evidence of relationship to strains obtained from another source was encountered.

<sup>12</sup> Jour. Am. Med. Assn., 1919, 72, p. 1542.

<sup>13</sup> Jour. Exper. Med., 1920, 32, p. 665.

The influenza bacillus is a representative of a heterogeneous group of organisms possessing the common property of requiring hemoglobin for growth but differing in their antigenic properties, although immunologically identical strains may occur in the same patient and have been found in 27% of a small series of cases.

These results lend no support to the view that the influenza bacillus of Pfeiffer is the cause of epidemic influenza.

# THE EFFECTS OF HEMOLYTIC STREPTOCOCCI ON THE BLOOD AND HEMOPOIETIC ORGANS OF RABBITS

PLATES 1 AND 2

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The present study was undertaken in order to throw further light, if possible, on the changes that may be caused by hemolytic streptococci on the blood and hemopoietic organs of rabbits.

In rabbits, the lymphocytes and basophile leukocytes occur in a higher percentage than in man. The granules of leukocytes stain with both basic and acid dyes and are highly refractive, and cells containing such granules are known as amphophiles and they correspond to the neutrophils in man. The total number of leukocytes in the blood of normal rabbits as estimated by Brinkerhoff and Tyzzer<sup>1</sup> was from 6,400 to 13,400 per cmm. My counts in 10 normal rabbits average 9,600 leukocytes and 4,682,000 erythrocytes. The percentages of different types of leukocytes obtained by various observers are given in table 1.

TABLE 1  
PERCENTAGES OF LEUKOCYTES OBTAINED BY VARIOUS OBSERVERS

	Brinkerhoff and Tyzzer, <sup>2</sup> Percentage	Bunting, <sup>3</sup> Percentage	The Author, Percentage
Amphophiles.....	40-50	53.5	40
Basophiles.....	4-8	8.8	6
Eosinophiles.....	0.5-1	0.5	0.5
Lymphocytes.....	45-55	53.5	48
Large mononuclears.....	2-8	7.1	5.5

It is unnecessary to deal with the structure of the marrow of rabbits in detail at this time as Muir,<sup>4</sup> Dickson,<sup>5</sup> and Brinkerhoff and Tyzzer<sup>6</sup> have described it fully. Normally the marrow of rabbits is more cellular than that of man and besides fat cells and supporting tissue it

Received for publication March 28, 1921.

<sup>1</sup> Jour. Med. Research, 1902, 7, p. 191.

<sup>2</sup> Ibid., p. 173.

<sup>3</sup> Univ. of Penn. Med. Bull., 1903, 16, p. 200.

<sup>4</sup> Jour. Path. & Bacteriol., 1901, 7, p. 161

<sup>5</sup> The Bone Marrow, a Cytological Study, 1908.

<sup>6</sup> Jour. Med. Research, 1905, 8, p. 449.

consists of normoblasts, megaloblasts, myeloblasts, myelocytes of various types and megakaryocytes. The granules of the myelocytes are amphophile.

The structure of the spleen and lymph nodes does not differ much from that of man except, as Ehrlich<sup>7</sup> noticed, nucleated erythrocytes may occur in the spleen, hence this organ in the rabbit may form erythrocytes during postembryonic life.

Rabbits in good condition and as nearly as possible of the same age and weight were inoculated intravenously with varying amounts of 24-hour dextrose broth cultures of typical hemolytic streptococci (Beta type). The experiments were repeated two or three times in every case in order to secure reliable results. The blood was examined at least once before injection, twice within the first 8 hours after, and thereafter once or twice a day as condition might demand. In making the differential counts, 500 leukocytes were counted. The red cells were counted by means of the Levy hemocytometer and the hemoglobin estimated by the Dare method. The number of nucleated erythrocytes and of degenerated leukocytes was estimated by the number seen in counting 500 leukocytes. The condition of the erythrocytes was studied according to Schleip's<sup>8</sup> method: A drop of blood is diluted with physiologic salt solution and then allowed to run in between two cover glasses, one larger than the other; cedar oil or vaseline is applied around the edge of the hollow of a hanging drop slide, and the cover glasses placed in such a position that the small one lies just inside of the hollow and the larger in a perfect contact with the oil. By this means mechanical injury to the cell may be avoided. The dried blood films were stained with various methods, but the best result was obtained with Jenner's stain. The marrow, spleen and lymph nodes were fixed in Zenker's fluid, embedded in paraffin, and sections, five microns thick, stained with hemotoxylin and eosin or Jenner's blood stain. Marrow films were stained by the Mallory and Wright methods.<sup>9</sup>

The following experiments will serve to illustrate the results obtained and the method of procedure.

*Exper. 1.*—Rabbit A received 3 c.c. of culture of a hemolytic streptococcus isolated from the normal throat and rabbit B the same amount of culture of a strain from the throat of a scarlet fever patient. Both animals were killed on the tenth day. The blood picture showed no special features except rabbit B had a high percentage of amphophile leukocytes with slender ribbon-like nuclei in the form of a curve, the significance of which will be considered later.

<sup>7</sup> Quoted by Brinkerhoff, *Ibid.*, p. 446.

<sup>8</sup> *Atlas of Haematology*, p. 10.

<sup>9</sup> *Pathological Technique*, 1918, p. 153.

TABLE 2  
EXPER. 1, RABBIT B

	Total Number Leuko- cytes	Percentage							
		Ampho- philes	Baso- philes	Eosino- philes	Lympho- cytes	Large Mono- nu- clears	Transi- tionals	Myelo- cytes	Degen- erated Ampho- philes
Before inocula- tion.....	12,420	42.0	5.6	1.4	49.0	2.0	0	0	0
4 hours after..	8,400	27.4	4.0	0	64.6	4.0	0	0	0
8 hours after..	3,500	28.6	2.4	0	60.2	8.8	0	0	0
24 hours after..	19,200	75.0	0	0	19.2	5.8	0	0	0
48 hours after..	21,620	78.4	0	0	20.0	1.6	0	0	0
72 hours after..	18,400	76.0	0	0	16.8	2.2	5.0	0	0
96 hours after..	16,200	65.4	0	0	23.0	4.6	7.0	0	0
5 days after...	19,300	72.6	2.0	0	15.0	6.2	4.2	0	0
6 days after...	17,975	65.4	3.2	0	20.4	6.4	4.6	0	0
7 days after...	23,075	66.2	2.0	0	12.4	9.4	10.0	0	0
8 days after...	17,600	59.2	5.0	0	32.0	3.8	0	0	0
9 days after...	14,550	44.4	4.0	0	39.4	2.2	0	0	0

*Exper. 2.*—The rabbit received 3 cc of broth culture of a streptococcus strain isolated from a case of cerebrospinal meningitis. The animal died with leukopenia associated with the presence of myelocytes. Degenerated leukocytes were found on the second day of infection.

TABLE 3  
EXPER. 2

	Total Number Leuko- cytes	Percentage							
		Ampho- philes	Baso- philes	Eosino- philes	Lympho- cytes	Large Mono- nu- clears	Transi- tionals	Myelo- cytes	Degen- erated Ampho- philes
Before inocula- tion.....	1,065	39.2	6.0	0	48.0	6.8	0	0	0
4 hours after..	4,425	24.0	5.6	0	64.4	6.0	0	0	0
8 hours after..	5,640	21.4	6.2	0	66.8	5.6	0	0	0
24 hours after..	21,240	76.2	0.2	0.4	18.6	0.4	4.2	0	5.3
48 hours after..	25,400	75.4	2.0	0	10.0	8.0	6.6	0	8.2
72 hours after..	3,125	26.6	0.8	0	41.0	9.2	4.0	6.4	3.0

*Exper. 3.*—A rabbit received 3 cc of culture of a streptococcus strain from the marrow of the rabbit in exper. 2. The blood picture of this animal is similar to the one in exper. 2, except that the percentage of degenerated leukocytes was much higher.

TABLE 4  
EXPER. 3

	Total Number Leuko- cytes	Percentage							
		Ampho- philes	Baso- philes	Eosino- philes	Lympho- cytes	Large Mono- nu- clears	Transi- tionals	Myelo- cytes	Degen- erated Ampho- philes
Before inocula- tion.....	9,460	50.0	6.0	0	42.6	1.4	0	0	0
4 hours after..	4,220	34.0	4.2	0	50.8	10.0	0	0	0
8 hours after..	6,250	36.0	1.4	0	52.6	10.0	0	0	0
24 hours after..	19,420	72.4	4.6	0	14.2	8.8	0	0	7.2
48 hours after..	27,450	82.6	4.4	0	8.2	1.8	3.0	0	11.2
72 hours after..	15,200	50.2	0.8	0.2	20.4	14.2	7.4	5.0	12.3
96 hours after..	7,625	32.4	2.4	0	46.2	6.0	5.2	8.8	9.5

*Exper. 4.*—A rabbit received 3 cc of culture of a streptococcus strain from a case of bronchopneumonia after it had been passed through mice 16 times. The degeneration of leukocytes was most marked on the third day and leukopenia developed on the day the animal died.

TABLE 5  
EXPER. 4

	Total Number Leuko- cytes	Percentage							
		Ampho- philes	Baso- philes	Eosino- philes	Lympho- cytes	Large Mono- nuclears	Transi- tionals	Myelo- cytes	Degen- erated Ampho- philes
Before inocula- tion.....	11,040	42.2	6.8	0	50.4	0.6	0	0	0
4 hours after..	7,460	29.0	1.0	0	34.6	15.4	0	0	0
8 hours after..	3,600	28.2	0.8	0	59.4	11.6	0	0	0
24 hours after..	21,200	68.2	0.8	0	22.8	8.2	0	0	8.2
48 hours after..	24,040	74.0	2.0	0	19.4	4.6	0	0	11.7
72 hours after..	16,200	42.4	2.4	0	24.6	7.2	9.2	14.2	18.3
96 hours after..	6,425	29.0	5.4	0	35.0	9.2	5.8	15.6	8.4

TABLE 6  
EXPER. 5

	Total Number Leuko- cytes	Percentage							
		Ampho- philes	Baso- philes	Eosino- philes	Lympho- cytes	Large Mono- nuclears	Transi- tionals	Myelo- cytes	Degen- erated Ampho- philes
Before inocula- tion.....	11,400	37.8	4.4	0.6	47.2	10.0	0	0	0
4 hours after..	9,250	29.0	1.0	0	64.2	5.8	0	0	0
8 hours after..	4,265	22.0	4.0	0	61.0	13.0	0	0	0
24 hours after..	14,200	57.0	4.0	0	24.0	9.6	5.4	0	27.5
48 hours after..	1,040	47.2	6.0	0	23.8	9.2	9.8	4.0	25.2
72 hours after..	4,250	34.6	4.2	0	48.4	4.8	0.6	7.4	20.5

TABLE 7  
EXPER. 6

	Total Number Leuko- cytes	Percentage							
		Ampho- philes	Baso- philes	Eosino- philes	Lympho- cytes	Large Mono- nuclears	Transi- tionals	Myelo- cytes	Degen- erated Ampho- philes
Before inocula- tion.....	10,620	45.0	4.0	0	47.2	3.8	0	0	0
4 hours after..	4,725	21.0	0	0	69.4	9.6	0	0	0
8 hours after..	5,675	27.2	0	0	59.6	13.2	0	0	0
24 hours after..	14,825	60.2	2.0	0	22.2	5.6	0	0	0
48 hours after..	18,020	52.8	4.0	0	35.2	8.0	0	0	0
72 hours after..	17,400	60.6	5.2	0	26.2	8.0	0	0	0
96 hours after..	16,325	57.0	2.0	0	32.8	5.2	3.0	0	0
5 days after...	13,000	54.0	4.8	0	32.0	3.0	6.2	0	0
6 days after...	14,025	56.2	6.4	0	26.6	6.0	4.8	0	0
7 days after...	21,050	68.2	3.0	0	21.6	2.2	5.0	0	0
8 days after...	25,625	78.4	1.0	0	19.0	1.0	0.6	0	0
9 days after...	24,040	81.0	0	0	16.8	0.2	2.0	0	0
10 days after...	22,040	72.4	0	0	23.0	0.6	4.0	0	0
11 days after...	16,675	72.6	2.0	0	15.0	3.0	7.4	0	0
12 days after...	15,940	65.0	0	0	29.2	2.2	3.6	0	0
13 days after...	26,000	76.2	2.0	0	12.4	3.0	2.4	4.0	4.2
14 days after...	14,500	65.4	3.2	0	10.4	5.0	2.8	13.2	0
15 days after...	8,842	46.2	2.0	0	32.4	2.4	5.0	12.0	0

*Exper. 5.*—A rabbit received 3 cc of culture of a streptococcus strain from the heart of a woman who died of puerperal sepsis. The largest number of leukocytes was 14,200 and the degeneration of leukocytes was more marked than in any other case.

*Exper. 6.*—A rabbit received 1 cc of a virulent streptococcus culture every 6 days; the animal died on the 16th day. The blood smears showed no degenerated leukocytes and myelocytes appeared in the peripheral circulation on the 13th day, but the former disappeared on the following day.

*Exper. 7.*—A series of 5 rabbits were inoculated with nonvirulent hemolytic streptococci in small doses every 6 days, and by this method I was able to produce a condition of anemia in two, one (C) giving 2,844,090 erythrocytes and 26% of hemoglobin, and the other (D) 3,046,000 erythrocytes and 28% of hemoglobin. Anisocytosis appeared earlier than poikilocytosis which was first noted at the end of the 4th week in rabbit C. The normoblasts as a rule appeared in the peripheral circulation as early as on the 2nd week after injection, but their number never was above 50 in counting 500 leukocytes. The erythrocytes of both animals after the 5th week showed diminished and uneven hemoglobin distribution and polychromatophilia, some of the cells containing minute black deposits at the periphery. No megaloblasts were found in the blood films. The marrow showed hyperplasia of normoblasts, and otherwise it was normal. The spleen showed a marked congestion and a large number of phagocytic cells crowded with blood pigments.

#### DISCUSSION

*Leukocytes.*—The animals inoculated with fatal doses of virulent hemolytic streptococcus usually showed degenerative changes in the amphophile leukocytes on the second day. Careful study showed that the principal and foremost change occurred in the nuclei of the amphophile leukocytes, in the form of swelling and disintegration. In some cases fragmentation and condensation of the nuclei were found. The cytoplasm was also abnormal: There was early an increase in the size of the granules and of the cell body itself, most of the swollen leukocytes having only a few poorly stained granules on a pale or pink background. In *exper. 2*, vacuoles in the cytoplasm of the amphophile leukocytes were noted the day before death. Schleip<sup>8</sup> states that vacuolar degeneration of leukocytes may occur in much debilitated or moribund patients some hours before death, and this condition he regards as a bad prognostic sign. The largest number of degenerated leukocytes was found in *exper. 5*. This animal did not have a high leukocyte count.

Arneth<sup>11</sup> regards the nuclear segments as representing the age of the cells, and when cells with one or two segments become more numerous than normal ("deviation to the left"), it is supposed to signify an impending exhaustion of the marrow. He divides neu-

<sup>11</sup> Die neutrophilen weissen Blutkörperchen, Jena, 1904, p. 17.

trophile leukocytes into five classes according to the number of nuclear segments. Schilling<sup>12</sup> separates neutrophile leukocytes into segmented nuclear cells, rod-nuclear cells, juvenile cells, and myelocytes and concludes that deviation to the left may mean either regeneration or exhaustion of the marrow, and deviation to the right a degeneration of leukocytes. Nagao<sup>13</sup> found that the Arneth index became disarranged immediately after the injection of killed nonhemolytic streptococci in guinea-pigs, reaching its maximum in about 3 hours, and then gradually returning to normal. Schilling states that the number of nuclear segments has no relation to the age of the cells and Neumann<sup>14</sup> calls attention to the fact that polymorphonuclear leukocytes become simple when they ceased their ameboid movement. My study shows that the nuclei which were undergoing degeneration offer a good deal of difficulty in the recognition of nuclear segments. In experiments 1 and 6 the amphophile leukocytes with a slender ribbon-like nucleus in the form of a curve occurred in a higher percentage than in the other cases. These cells correspond morphologically to the cells with "tiefeinge-buchtetem Kerne" according to Arneth and an increase in the number of these cells should mean a deviation to the left and be a bad prognostic sign, but the experiment indicates the contrary. However, the appearance of myelocytes especially when associated with leukopenia seems to be a danger signal from the data thus far obtained. In view of what has been said, Schilling's deduction seems to be more valid, and clinical application of the original Arneth index should be made with reservation.

*The Marrow.*—In acute streptococcus infection Dickson found in the marrow hemorrhage and a large number of phagocytic cells which were crowded with pigments in one case, in another case there was a marked necrosis of the marrow cells. He also described "gelatinous degeneration" of the marrow in acute and chronic cases. As to the significance of this gelatinous degeneration which bears on the leukoblastic reaction, Muir<sup>15</sup> holds that this change may interfere with cellular hyperplasia. Lucibelli<sup>16</sup> found that loss of straining property, irregular distribution of chromatin, swelling and solution of the nuclei of the marrow cells followed fatal injection of typhoid, paratyphoid, and colon bacilli. Muir<sup>17</sup> found the same changes in experimental staphylococcus infection.

<sup>12</sup> Quoted by Gruner: *The Biology of Blood-Cells*, 1913, p. 217.

<sup>13</sup> *Jour. Infect. Dis.*, 1920, 27, p. 22.

<sup>14</sup> Quoted by Gruner: *The Biology of Blood Cells*, 1913, p. 216.

<sup>15</sup> *Trans. of the Path. Soc., London*, 1902, 53, p. 379.

<sup>16</sup> Quoted by Gruner, *The Biology of Blood-Cells*, 1913, p. 199.

<sup>17</sup> *Jour. Path. & Bacteriol.*, 1901, 7, p. 161.



In my experiments hyperplasia in the marrow usually occurred after inoculation of streptococci regardless of the virulence or number of organisms inoculated. In the early stage of virulent streptococcus infection, the cytoplasm of myelocytes presented a granular appearance and stained more strongly with eosin. In general, the degenerative changes of the marrow cells resulting from infection with virulent hemolytic streptococci in rabbits consisted of karyorrhexis, karyolysis, vacuolation and solution of cytoplasm. The microphotographs give a better idea of the change than an extended verbal description would. In exper. 5 the marrow consisted mostly of myeloblasts, and all the leukoblastic cells were undergoing degeneration especially at the periphery of the marrow. In exper. 6 the marrow presented the gelatinous degeneration described by Dickson, and only a few marrow cells were seen here and there in a pink homogeneous material. A rabbit killed on the second day of virulent streptococcus infection had a similar change in the marrow, but the areas were small and consisted mostly of delicate fibrils running in all directions, forming an interlacing network. Strange to say, this picture is practically identical to that described by Dickson in a case of ulcerative endocarditis of less than three weeks' duration.

The megakaryocytes seem to be very sensitive to toxic action as they undergo degeneration in rabbits inoculated with hemolytic streptococci of only slight virulence. In no case could the three protoplasmic zones be made out. The cytoplasm had a strong affinity for eosin, and the nuclei were swollen, fragmented, poor or rich in chromatin. These cells usually were present in large numbers within the first 48 hours after injection, and then gradually decreased in number as the course of infection advanced. Within them erythrocytes, myelocytes, and amphophile leukocytes were found. The phagocytic cells described by Dickson were noted in several cases, especially when there was hemorrhage in the marrow.

*The Spleen.*—Evans<sup>18</sup> showed that streptococci may produce acute splenic swelling of a gray type characterized by less congestion and hyperplasia of the cells of myeloid type, and he argues that there is no hyperplasia of the reticuloendothelial cells in contrast with the red type because the leukocytes then are still in function. Muir found a hyperplasia of phagocytic cells in spleen in experimental staphylococcus infection. Nagao<sup>19</sup> produced a marked degeneration of the cells in the malpighian bodies by the injection of nonhemolytic streptococci.

<sup>18</sup> Johns Hopkins Hosp. Bull., 1916, 27, p. 356.

<sup>19</sup> Jour. Infect. Dis., 1920, 27, p. 22.

In exper. 2 the spleen presented a marked congestion and hemorrhage, and some of the erythrocytes were poor in hemoglobin and others appeared in different shapes. A large number of phagocytic cells were crowded with blood-pigments and Flemming's bodies and the latter were more numerous in exper. 5 than in any other case. By many Flemming's bodies are considered to be derived from degenerated lymphocytes, but after a careful study I believe that some of them come from degenerated polymorphonuclear leukocytes. Nucleated erythrocytes and myelocytes were found practically in every case. The Malpighian bodies were usually decreased in size, and in exper. 3 a necrotic change ("focal necrosis") occurred in the center.

The lymph glands presented no special features. Congestion and slight hemorrhage were seen in certain acute cases and leukocytic infiltration occurred in every case. Foci of necrosis of the lymph follicles were found in exper. 3.

*Leukocytosis and Leukopenia.*—Leukocytosis and leukopenia have been explained on the basis of chemotaxis and reaction on part of the marrow. In leukocytosis the explanation rests on a good evidence, but the origin of leukopenia is still a matter of speculation, and its development, in spite of the presence of chemotactic substances, is hard to understand. The view that leukopenia is due to an exhaustion of the marrow seems reasonable and the question arises whether a normal marrow can become exhausted without a preliminary leukoblastic reaction. If not, what other possibilities are there that exhaustion of marrow that eventually can give rise to leukopenia may be produced? To answer this question it is necessary to study the condition of marrow as well as that of blood. During the course of my experiments two types of leukopenia were observed in severe infections: the one occurring immediately after inoculation and the other some time later. The former seems to be largely due to an abnormal distribution of leukocytes, while the latter probably is due to the retrogressive changes of amphophile leukocytes in the circulation and of marrow. This is illustrated especially by exper. 5.

*Streptococcus Leukocidin.*—Hektoen<sup>20</sup> found that the culture fluids of virulent streptococci may not only decrease the phagocytic activity of leukocytes, but also cause swelling and arrest of ameboid movement and grave morphologic changes, and M'Leod<sup>21</sup> noted a retrogressive change of the amphophile leukocytes in the heart blood of

<sup>20</sup> Jour. Am. Med. Assn., 1906, 46, p. 1407.

<sup>21</sup> Jour. Path. & Bacteriol., 1915, 19, p. 392.

rabbits that died of streptococcus infection. Ruediger<sup>22</sup> and Nakayama<sup>23</sup> brought to light the toxic effect of virulent streptococci on leukocytes by means of the methylene blue reduction test, leukocytes acted on by streptococcus filtrates failing to reduce the blue.

By the smear method I tested the leukocytal action of the strain of hemolytic streptococcus used in exper. 4. The organism was cultivated in serum broth for 24 hours, and the filtrate which had been passed through a Massen filter was used to test for leukocidin. The leukocytes were obtained from the sterile peritoneal exudate of a rabbit produced by injection of aleuronat suspension. The marrow was removed from the shaft of a femur and suspended in salt solution and by shaking a heavy suspension of marrow cells could be obtained. The result is illustrated in table 8.

TABLE 8  
LEUKOCYTAL ACTION OF STRAIN OF HEMOLYTIC STREPTOCOCCUS USED IN EXPER. 4

Amount of Culture Filtrate	Leukocytes	Result	Marrow	Result
1 c c	1 c c	+	1 c c	+
0.75 c c	1 c c	+	1 c c	+
0.5 c c	1 c c	+	1 c c	+
0.25 c c	1 c c	0	1 c c	+
0.1 c c	1 c c	0	1 c c	0
Serum broth	1 c c	0	1 c c	0
Salt solution	1 c c	0	1 c c	0

As a rule, after one hour's incubation, at 37C, of the mixture of leukocytic suspension and streptococcus filtrate the amphophile leukocytes became swollen, the cytoplasm granular, staining with methylene blue, and in certain cases undergoing solution. The nuclei also became swollen and occasionally fragmented. In control specimens containing serum broth or salt solution the leukocytes remained normal as long as three hours. The streptococcus filtrate practically exerted the same effect on marrow cells in suspension, but the degenerative changes appeared much earlier. The changes resembled altogether those that occurred *in vivo*. It is noteworthy that culture filtrates injected intravenously by me did not cause any toxic effect on the leukocytes in the peripheral circulation and only a slight hyperplasia of marrow. This may be because the toxin was not concentrated enough to produce a massive effect, and because degenerated leukocytes, if any were produced, were taken out of the peripheral circulation promptly.

<sup>22</sup> Jour. Am. Med. Assn., 1905, 44, p. 198.

<sup>23</sup> Jour. Infect. Dis., 1920, 27, p. 86.

## CONCLUSIONS

Hemolytic streptococci may produce a toxic substance that causes degeneration of leukocytes and marrow cells *in vitro*.

In rabbits intravenous injections of virulent hemolytic streptococci produce retrogressive changes in the amphophile leukocytes in the peripheral circulation and in the marrow cells. This change is probably due to the same substance that acts on the cells *in vitro*, but of course the disintegrated products of streptococci may have the same effect. This degeneration in amphophile leukocytes and marrow cells is the factor that may give rise to leukopenia.

Hemolytic streptococci of low virulence produce no definite morphologic changes in the amphophile leukocytes *in vitro* or *vivo*, but cause a disarrangement of the Arneth index to the left.

Hemolytic streptococci may produce gelatinous degeneration of marrow.

Hyperplasia of phagocytic cells in spleen may take place in hemolytic streptococcus infection.

By subacute infection with small doses of nonvirulent hemolytic streptococci, a condition of advanced anemia may be produced (exper. 7). Since there is a lower hemoglobin content, an absence of megaloblasts and no marked lesions in the marrow, the anemia is of secondary type.

## EXPLANATION OF PLATES 1 AND 2

Fig. 1.—Rabbit leukocytes after being acted on by culture filtrate of virulent hemolytic streptococcus; X 1000.

Fig. 2.—Rabbit leukocytes acted on by virulent streptococcus culture; X 1000.

Fig. 3.—Blood smear on the second day of infection showing degenerated amphophiles; exper. 5, Jenner's stain; X 1000.

Fig. 4.—Marrow smear, exper. 3, showing degenerated marrow cells; X 1000.

Fig. 5.—Section of marrow, exper. 5, showing marked degeneration; X 1000.

Fig. 6.—Gelatinous degeneration of marrow in early stages of acute infection; exper. 3; X 1000.

Fig. 7.—Advanced gelatinous degeneration of marrow; exper. 6; X 500.

Fig. 8.—Section of spleen showing several phagocytic cells crowded with erythrocytes and black deposits; exper. 6; X 1000.

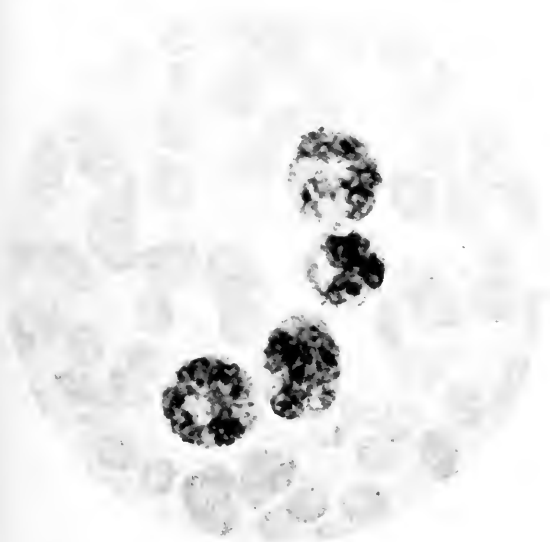
PLATE 1



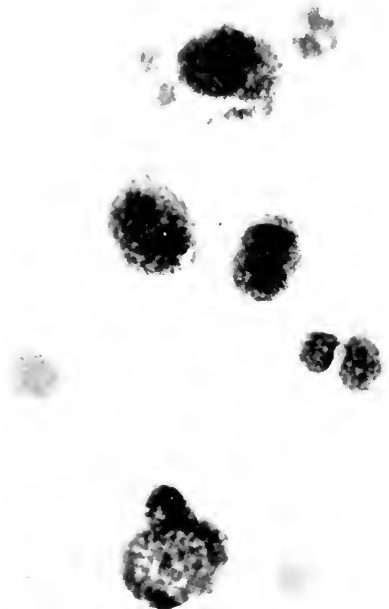
2



1

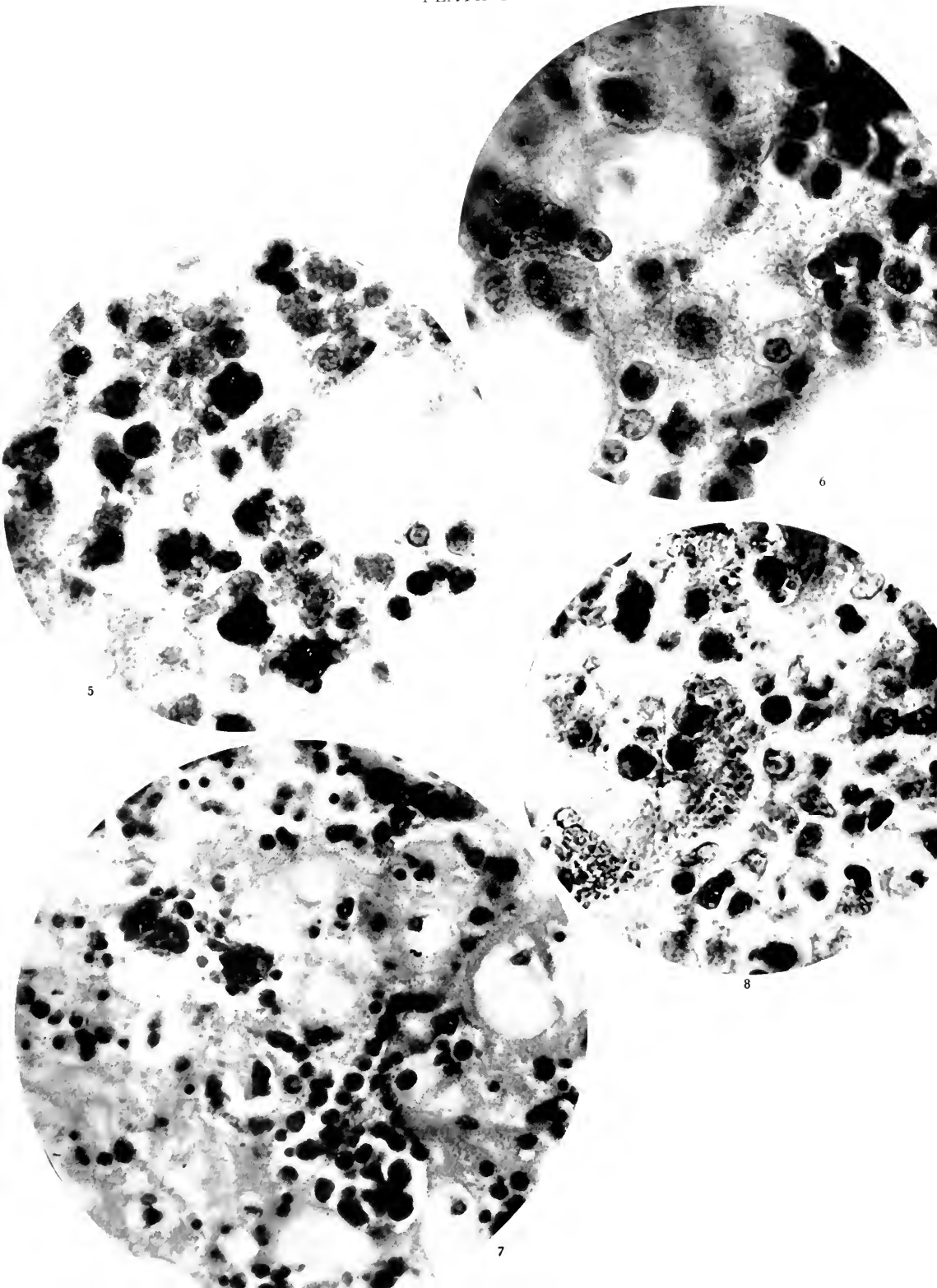


3



4

PLATE 2



# REACTIONS OF THE NASAL CAVITY AND POSTNASAL SPACE TO CHILLING OF THE BODY SURFACE

## II. CONCURRENT STUDY OF BACTERIOLOGY OF NOSE AND THROAT

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By following the surface temperatures of skin and mucous membranes by means of thermopiles held in apposition with them, it has been possible to show that chilling of the body surface causes reflex vasoconstriction and diminished blood supply to the skin, palate and oropharynx,<sup>1</sup> the palatine tonsils,<sup>2</sup> and the nasal cavity and postnasal space.<sup>3</sup> Concurrently with these experiments, the bacteriology of the throats and nasal cavities of the subjects have been studied in the hope of obtaining information regarding: first, the normal flora of healthy young men; and, second, any possible changes induced in that flora by the experimental procedure.

Bacteriologic data concurrent with the experiments in the nasal cavity and postnasal space follow. We would explicitly state again as we have before,<sup>2</sup> however, that the fluctuations in bacterial flora noted cannot with certainty be referred to the influence of the experiments with which they were correlated in time, nor can the influence of the experiments be attributed solely to the chilling involved. The protocols are given in some detail so that the evidence can better be evaluated.

*Material and Method.*—The medium employed was a 5% rabbit-blood meat-infusion agar. Cultures were taken from both sides of the nasal cavity, the right tonsil and the posterior wall of the oropharynx by separate swabs. The vestibule of the nose was wiped off with wet cotton before the nasal cultures were taken. Each swab was immersed in sterile broth and then applied to the blood-agar plate; the remaining area of the plate was inoculated by a platinum loop. Cultures were incubated for 36 hours. A number of colonies were then counted over

Received for publication April 7, 1921.

<sup>1</sup> Mudd, S., and Grant, S. B.: Jour. Med. Res., 1919, 40, p. 53.

<sup>2</sup> Grant, S. B.; Mudd, S., and Goldman, A.: Jour. Exper. Med., 1920, 32, p. 87.

<sup>3</sup> Mudd, S.; Goldman, A., and Grant, S. B.: Jour. Exper. Med., 1921, 34, No. 1.

a portion of the plate, and the nature of each determined. Pneumococcus and nonhemolytic streptococcus were put in the same group because of the difficulty in differentiating these by their morphology.

*Results.*—Four subjects were used in this study. Cultures were made daily (with a few omissions), from June 21 to July 19, from 2 subjects, S. M. and A. G. Cultures were similarly made from the third, S. B. G., from June 21 to June 30, inclusive. Cultures were made from the fourth, F. J. C., only once. S. M., A. G. and F. J. C. each developed a mild coryza. S. B. G. was unaffected.

Subject S. M. showed in the nose from the outset *Staphylococcus albus*. On June 24 and thereafter, *S. aureus* also was found. Diphtheroids appeared in cultures from each nasal cavity made 4 hours after his third intranasal experiment. *S. albus* remained throughout all the experiments. *S. aureus* was present occasionally, but, except on the days following its first appearance, in smaller numbers than *S. albus*. The diphtheroids fluctuated in numbers and were sometimes absent. There was no apparent relation between their numbers and the chilling.

The tonsil contained at the outset nonhemolytic streptococcus, pneumococcus and *S. albus*. Streptococcus hemolyticus appeared in the tonsil culture taken 24 hours after the first experiment, on June 22; the applicator in this experiment was on the nasal septum, and the subject's mouth was closed. Forty-eight hours after the experiment, a pure culture of *S. hemolyticus* appeared on the tonsil plate. The number diminished on the following day to 11% of all colonies, and subsequently remained present in numbers 1 to 12% of all colonies counted, through July 12. Nose and throat remained clinically normal throughout this time. Further experiments were performed on this subject July 12, 15 and 17, with the thermopile tips respectively on the nasopharyngeal and oropharyngeal wall and in the air of the postnasal space. The proportion of hemolytic streptococci in the tonsil cultures slowly rose during this time—July 12, 12%; July 14, 16%; July 16 and 17, numerous, not counted; July 19, 25%; July 20, 36%. July 17, this subject began to develop symptoms of coryza. By July 19 he had cough, nasal stuffiness and rhinorrhea and malaise. Symptoms were present but abated the following day. This subject's mother had had a severe cold since about July 12; his symptoms may or may not have been connected with the experiments.

The pharynx showed nonhemolytic streptococcus and pneumococcus, and occasionally *S. albus* and *S. aureus*. June 27 *S. hemolyticus*



appeared and subsequently it was obtained in 4 cultures, each time associated with tonsil cultures containing a similar organism. *S. hemolyticus* is so usual an inhabitant of the tonsils<sup>4</sup> that its incidence here may or may not have been connected with the experiments.

Subject A. G. showed in the nasal cavity initially *S. aureus*; subsequently *S. albus* appeared on each side. The right side showed usually a preponderance of *S. albus*, the left of *S. aureus*. No other organisms appeared in the nasal cultures.

The right tonsil showed a nonhemolytic streptococcus throughout, i. e., from June 21 to July 19. A. G. was the subject June 21, 23, and 24; application was made on the anterior end of the right nasal septum, the anterior end of the left lower turbinate, and in the left middle meatus, respectively. The symptoms of a slight rhinitis—nasal stuffiness, slight headache and slight mucopurulent discharge—developed June 24. The secretion and stuffiness persisted until June 29. June 24, two colonies of *M. catarrhalis* appeared on the tonsil plate, and June 26 one on the tonsil and three on the pharynx plate. June 28 *S. Albus* began to be present in the right nose. Otherwise no change in the bacteriologic condition was noted, the nose showing *S. aureus* and the tonsil and pharynx nonhemolytic streptococci as before. A. G. was again the subject July 7 and 17, with the applicator on the right middle turbinate in the first case and with no mucous membrane application in the second. July 17, twenty colonies (44%) of *M. catarrhalis* appeared on the tonsil plate. There were no accompanying clinical symptoms. *S. hemolyticus* appeared after the experiment of July 7, four colonies on the tonsil and one on the pharynx plate on July 7, one on the tonsil plate July 8, and one on the tonsil plate July 12. These were the only appearances of hemolytic streptococci in this subject either in the series of 1919 or of 1920.

In subject S. B. G. there were present in the nose *S. aureus* and *S. albus*. On the right tonsil there were a nonhemolytic streptococcus, pneumococcus, *S. aureus* and *S. albus*. The pharynx showed a nonhemolytic streptococcus and pneumococcus. There was practically no change in the bacterial flora throughout the period studied, nor were there any signs of a cold. The cultures from the pharynx were frequently sterile and always showed relatively few bacteria, as was the case in this subject in 1919.<sup>2</sup>

F. J. C., whose pharyngeal culture showed abundant hemolytic streptococci, developed a mild cold the day following his first experi-

<sup>4</sup> Davis, D. J.: Jour. Am. Med. Assn., 1920, 74, p. 317 and 75, p. 792.

TABLE 1  
SUBJECT S. M. BACTERIOLOGY OF NASAL CAVITY, TONSIL AND PHARYNX

Inoculation	Place Cultivated	Nonhemolytic Streptococci and Pneumococci		S. hemolyticus		S. aureus		S. albus		Diphtheroids		Remarks
		Number of Colonies Counted	Per-centage of All Colonies	Number of Colonies Counted	Per-centage of All Colonies	Number of Colonies Counted	Per-centage of All Colonies	Number of Colonies Counted	Per-centage of All Colonies	Number of Colonies Counted	Per-centage of All Colonies	
e 21 . m.	Right side of nose...	0	0	0	0	0	0	..	100	0	0	Subject of exper. 6, 9:15 to 10:21 a. m. Application on right nasal septum
	Left side of nose...	0	0	0	0	0	0	..	100	0	0	
	Right tonsil...	20	67	0	0	0	0	10	33	0	0	
	Pharynx...	..	100	0	0	0	0	0	0	0	0	
e 22* . m.	Right side of nose...	0	0	0	0	0	0	0	0	0	0	Nose and throat normal. Subject of exper. 8, 3:40 to 4:57 p. m. Application on right inferior turbinate
	Left side of nose...	0	0	0	0	0	0	0	0	0	0	
	Right tonsil...	..	100	0	0	0	0	0	0	0	0	
	Pharynx...	..	100	0	0	0	0	..	100	0	0	
e 23* . m.	Right side of nose...	0	0	0	0	0	0	..	100	0	0	Nose and throat normal
	Left side of nose...	0	0	0	0	0	0	..	100	0	0	
	Right tonsil...	7	88	1	12	0	0	0	0	0	0	
	Pharynx...	0	0	0	0	0	25	15	73	0	0	
e 24 . m.	Right side of nose...	0	0	0	0	5	100	15	0	0	0	Subject of exper. 25, 9:30 to 11:04 a. m. Application in right inferior meatus. Swab at 2 p. m. brought away blood from right side of nose
	Left side of nose...	0	0	0	0	0	0	0	0	0	0	
	Right tonsil...	0	0	0	0	0	0	0	0	0	0	
	Pharynx...	0	0	0	0	0	0	0	0	0	0	
e 25* . m.	Right side of nose...	..	100	0	0	0	0	0	0	0	0	Slight redness of right turbinates. Otherwise nose and throat normal
	Left side of nose...	0	0	0	0	0	0	0	0	0	0	
	Right tonsil...	100	89	12	11	0	0	11	63	3	14	
	Pharynx...	..	100	0	0	0	0	0	0	0	0	
e 26 . m.	Right side of nose...	0	0	0	0	10	29	5	14	20	57	Nose and throat normal. Subject of exper. 26, 11 a. m. to 1:04 p. m. Application in nasopharynx. Applicator ill-fitting: considerable trauma of nasopharynx
	Left side of nose...	0	0	0	0	20	64	1	3	10	32	
	Right tonsil...	..	100	1	1	0	0	0	0	0	0	
	Pharynx...	100 (app.)	99	0	0	0	0	0	0	0	0	
e 27* . m.	Right side of nose...	0	0	0	0	15	45	3	9	15	45	Right inferior turbinate slightly reddened
	Left side of nose...	0	0	0	0	0	0	0	0	0	0	
	Right tonsil...	29	88	4	12	0	0	3	13	4	17	
	Pharynx...	37	92	3	8	0	0	0	0	0	0	
e 28 . m.	Right side of nose...	0	0	0	0	3	13	20	87	0	0	Nose and throat normal
	Left side of nose...	0	0	0	0	20	57	15	43	0	0	
	Right tonsil...	30	79	1	3	0	0	7	18	0	0	
	Pharynx...	10	83	0	0	0	0	2	17	0	0	
e 29 . m.	Right side of nose...	0	0	0	0	0	0	..	100	0	0	Slight secretion from both sides of nose; otherwise nose and throat normal. Subject of exper. 15, 3 to 4:37 p. m. Application in nasopharynx
	Left side of nose...	0	0	0	0	40	95	2	5	0	0	
	Right tonsil...	45	96	2	4	0	0	0	0	0	0	
	Pharynx...	3	14	0	0	0	0	20	86	0	0	
e 30* m.	Right side of nose...	0	0	0	0	0	0	..	100	0	0	Nose and throat normal
	Left side of nose...	0	0	0	0	25	63	15	37	0	0	
	Right tonsil...	50	98	1	2	0	0	0	0	0	0	
	Pharynx...	0	0	0	0	0	0	0	0	0	0	
July 1	Right side of nose...	0	0	0	0	0	0	20	50	20	50	Nose and throat normal
	Left side of nose...	0	0	0	0	10	29	10	29	15	42	
	Right tonsil...	50	98	1	2	0	0	0	0	0	0	
	Pharynx...	53	96	1	4	0	0	0	0	0	0	



TABLE 2

SUBJECT A. G. BACTERIOLOGY OF NASAL CAVITY, TONSIL AND PHARYNX

Date	Place Cultivated	Nonhemolytic Streptococcus and Pneumococcus			S. hemolyticus			S. aureus			S. albus			M. catarrhalis			Undetermined			Remarks
		Number of Colonies Counted	Percentage of All Colonies	Number of Colonies Counted	Percentage of All Colonies	Number of Colonies Counted	Percentage of All Colonies	Number of Colonies Counted	Percentage of All Colonies	Number of Colonies Counted	Percentage of All Colonies	Number of Colonies Counted	Percentage of All Colonies	Number of Colonies Counted	Percentage of All Colonies	Number of Colonies Counted	Percentage of All Colonies	Number of Colonies Counted	Percentage of All Colonies	
21* m.	Right side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Nose and throat normal at time of culture. Subject of exper. 27, 12:45 to 1:45 p. m. Application on anterior part of right nasal septum
	Left side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Right tonsil...	23	92	0	0	0	0	0	0	0	0	0	0	0	0	2	8	0	0	
22 m.	Right side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Left side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Nose and throat normal
	Right tonsil...	32	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Pharynx...	80	80	0	0	0	0	8	20	0	0	0	0	0	0	0	0	0	0	
23* m.	Right side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Left side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Subject of exper. 2, 1:14 to 2:48 p. m. Application on anterior end of left lower turbinate body
	Right tonsil...	100	100	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	
	Pharynx...	100	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
24* m.	Right side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Left side of nose...	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Headache and nasal "stiffness." Right side of nose; mucopurulent secretion; inferior turbinate small. Left side of nose normal. Subject of exper. 9, 12:08 to 1:24 p. m. Application in left middle meatus
	Right tonsil...	24	80	0	0	0	0	4	13	0	0	0	0	2	7	0	0	0	0	
	Pharynx...	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
25 l.	Right side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Left side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Slight rhinitis. Swab of left side of nose brought away blood. Throat normal. Pharynx culture sterile
	Right tonsil...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Pharynx...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
26 m.	Right side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Left side of nose...	19	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Left side of nose has little breathing space. Turbinates smaller. Right side of nose normal. Dull pain in left eye. Throat normal. Nasal stuffiness. Voice has nasal twang
	Right tonsil...	40	95	0	0	0	0	0	0	0	0	0	0	1	5	0	0	0	0	
	Pharynx...	0	0	0	0	0	0	0	0	0	0	0	0	3	7	0	0	0	0	
28	Right side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Left side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Slight amount of secretion in both sides of nose. Right inferior turbinate almost occludes cavity. Left side of nose stuffy. Pharynx and tonsil normal. Nose and throat normal
	Right tonsil...	59	91	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Pharynx...	25	74	0	0	0	0	0	0	0	0	0	0	0	0	5	14	0	0	
29 m.	Right side of nose...	0	0	0	0	0	0	1	11	0	0	0	0	0	0	0	0	0	0	
	Left side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Slight amount of secretion in both sides of nose. Right inferior turbinate almost occludes cavity. Left side of nose stuffy. Pharynx and tonsil normal. Nose and throat normal
	Right tonsil...	40	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Pharynx...	100	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
30 n.	Right side of nose...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Left side of nose...	0	0	0	0	0	0	25	63	0	0	0	0	0	0	0	0	0	0	Nose and throat normal
	Right tonsil...	40	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Pharynx...	35	88	0	0	0	0	0	0	0	0	0	0	0	0	2	5	12	0	



ment (applicator on left nasal septum). Unfortunately a contagious origin cannot be ruled out, however, for his baby brother developed a cold about the same time.

#### PROTOCOLS OF EXPERIMENTS INCLUDED IN TABLES 1 AND 2

Subject S. M.: Mouth closed; nose breathing. 14 respirations per minute. For dates, times and sites of application of mucous membrane thermopile, see table 1.

*Exper. 6.*—Room temperature 17-18 C.; 0 to 15.5 minutes, wrapped; 15.5 to 18.5, unwrapped, no fan; 18.5 to 29.7, unwrapped, fan on back; 24, ampule of amyl nitrite inhaled; 27.9, begins shivering; 29.7 to 66, wrapped. During the experiment the subject felt pain at times at the site of application; otherwise no particular sensation; no rhinorrhea.

*Exper. 8.*—Room temperature about 17 C.; 0 to 12.5 minutes, wrapped; 12.5 to 19, unwrapped, no fan; 19 to 48, unwrapped, fan on; 21.5, begins shivering; 39 to 41.7, inhales amyl nitrite; 48 to 77, wrapped; 54, slight readjustment of mucous membrane thermopile in nose, followed by two hard sneezes, lacrimation and rhinorrhea. After the experiment secretion was seen in both nasal cavities.

Note by subject after experiment:

"Some feeling of soreness on swallowing which feels as though localized on right side of midline at level of thyroid cartilage; due to traumatism in violent sneezing with which experiment ended. Rest of afternoon and until retiring at 9:45 p. m. felt slight soreness on swallowing. Next morning felt normal."

This sneezing may have expressed an infected plug from a tonsillar crypt and have been responsible for the appearance of hemolytic streptococci in the surface tonsil culture made the next day.

*Exper. 25.*—Room temperature about 15 C.; 0 to 10 minutes, wrapped; 10 to 16, unwrapped, no fan; 16 to 28, unwrapped, fan on; 28 to 51.5, wrapped; 43.2 to 45.2, amyl nitrite inhaled; 52, mucous membrane applicator removed; 60 to 75.5, wrapped, no fan; 75.5 to 85.5, wrapped, fan on; 85.5 to 93.5, wrapped, no fan.

Note by subject: "Attempt at applying thermopile to middle turbinate; after one insertion it was withdrawn and was noted to bear one fleck of blood. Considerable pain, sneezing, lacrimation and rhinorrhea caused by this attempt.

"Application made by slowly slipping thermopile along floor of nose until it wedged apparently between floor and middle turbinate with applying surface felt to be against lateral wall presumably in inferior meatus. Considerable rhinorrhea and sneezing caused by this procedure."

*Exper. 26.*—Room temperature about 18 C.; 0 to 14 minutes, wrapped; 14 to 23.2, unwrapped, no fan; 23.2 to 31, unwrapped, fan on; movements of pharynx; 31 to 69, wrapped; 69 to 78, unwrapped, fan on; repeated swallowing; 76.5, begins shivering; 78 to 97, wrapped; 97 to 123.5, unwrapped, fan on; repeated swallowing; 101, begins shivering; 103, teeth chattering; 118.5 to 122.2, amyl nitrite inhaled. Inhalation of amyl nitrite in this experiment which typically seemed to cause mouth to fill up with saliva and necessitated repeated swallowing. Swallowing in this experiment was extremely painful, due to ill-fitting applicator.

*Exper. 15.*—Room temperature about 19 C.; 0 to 20 minutes, wrapped; 20 to 44, unwrapped, fan on; 44 to 67, wrapped; 55.5 to 59.2, inhales amyl nitrite;

repeated swallowing as typically; 67 to 81, unwrapped, fan on; 76, begins shivering; 81 to 97, wrapped.

*Exper. 14.*—Zero to 10.5 minutes, wrapped; 10.5 to 26.5, unwrapped, fan on; 26.5 to 50, wrapped; 33.5 to 36.5, inhales amyl nitrite; repeated swallowing; swallowing not painful.

*Exper. 18.*—Room temperature 18-19 C.; 0 to 10 minutes, wrapped; 10 to 28.5, unwrapped, fan on; 30 to 38, wrapped.

*Exper. 11.*—Room temperature 16-17 C.; 0 to 22.6 minutes, wrapped; 22.7 to 28.7, unwrapped, no fan; 28.7 to 39.3, unwrapped, fan on; 29.5, begins shivering; 39.3 to 65.4, wrapped; 47.2 to 50.5 inhales amyl nitrite, much swallowing; 65.4 to 71, unwrapped, fan on. Blood flecks on applicator tip when withdrawn. Throat felt sore rest of day; next day soreness gone.

*Exper. 22.*—Room temperature 18-19 C.; 0 to 13.5 minutes, wrapped; 13.5 to 21.5, unwrapped, no fan; 21.5 to 41, unwrapped, fan on; 22.5, began shivering; 41 to 50, wrapped; 41, applicator felt painful in nasopharynx.

*Exper. 21.*—Room temperature 18.5-18.8 C.; 0 to 15.5 minutes, wrapped; 15.5 to 23, unwrapped, no fan; 23 to 36, unwrapped, fan on; 25.5, begins shivering; 36 to 58, wrapped; 45 to 48.5, inhales amyl nitrite; repeated swallowing.

Subject A. G.: Mouth closed; nose breathing, 14 respirations per minute. For dates, times and sites of mucous membrane application, see table 2.

*Exper. 27.*—Room temperature about 17 C.; 0 to 32 minutes, wrapped; 32 to 57.5, unwrapped, fan on; 36, begins shivering; 57.5 to 60.5, wrapped.

*Exper. 2.*—Room temperature 16 to 17 C.; 0 to 12 minutes, wrapped; 12 to 19.2 minutes, unwrapped, no fan; 19.2 to 38, unwrapped, fan on; 23.2 to 26.2, inhaled amyl nitrite; 38 to 88.5, wrapped; 76.1 to 78.5, inhaled amyl nitrite; 88.5 to 94, unwrapped, fan on.

*Exper. 9.*—Room temperature 16.5-16.8 C.; 0 to 21 minutes, wrapped; 21 to 28.5, unwrapped, no fan; 28.5 to 46, unwrapped, fan on; 38.2 to 40.2, inhales amyl nitrite; 41.7 begins shivering; 46 to 76, wrapped.

Note by subject: "Considerable pain felt when applicator was first inserted, and slight pain throughout experiment."

*Exper. 28.*—Room temperature 20.5 to 20.8 C.; 0 to 19 minutes, wrapped; 19 to 26, unwrapped, fan on; 26 to 47, wrapped; 47 to 70, unwrapped, fan on; back moistened at intervals; 56.5 to 58.5, inhales amyl nitrite; 70 to 78.5, wrapped.

*Exper. 29.*—Room temperature about 19 C.; 0 to 16 minutes, wrapped; 16 to 26.5, wrapped, fan on; 26.5 to 40, unwrapped, fan on; 40 to 45.5, wrapped, fan on; 45.5 to 50, wrapped, fan off.

#### DISCUSSION AND SUMMARY

Nonhemolytic streptococcus was found in all 4 persons studied, *S. hemolyticus* in 3, pneumococcus in 2, and *M. catarrhalis* in one. In S. M. on one occasion there was an abundance of *S. hemolyticus* in the tonsil cultures before and during the symptoms of cold and sore throat. In F. J. C. a cold followed a single exposure of a person with abundant hemolytic streptococci. The cases of S. M. and F. J. C., taken with a considerably less doubtful instance in S. M. in 1919,<sup>2</sup> are suggestive,

though not conclusive, of experimental excitation of throat infection by *S. hemolyticus*. They are of interest as contributing evidence in corroboration of recent studies incriminating this organism in upper respiratory infections, e. g., Barnes,<sup>5</sup> Mathers,<sup>6</sup> Floyd,<sup>7</sup> Davis.<sup>4</sup>

The appearance of *M. catarrhalis* in A. G. after experimentation was paralleled by a like occurrence in the same subject in 1919,<sup>2</sup> and then, as in June, 1920, the appearance of this organism was to some degree correlated, in time at least, with clinical symptoms of infection.<sup>8</sup>

In our studies, as in that of Bloomfield,<sup>9</sup> the occurrence of non-hemolytic streptococci was practically constant in the throats of all subjects, and pneumococci, hemophils, and gram-positive cocci were found somewhat less frequently in both series. Gram-negative cocci were much less in evidence in our studies than in Bloomfield's. *Streptococcus hemolyticus* also was somewhat irregularly present in the tonsil and pharyngeal swab-cultures of both series, and in several instances was in correlation with infectious symptoms.

In the nasal cultures both of 1919 and 1920 practically no organisms other than *S. aureus* and *S. albus*, and in one case diphtheroids, were found.<sup>10</sup>

<sup>5</sup> The Tonsils, 1914, p. 67.

<sup>6</sup> Jour. Infect. Dis., 1917, 20, p. 1.

<sup>7</sup> Floyd, C.: Boston Med. and Surg. Jour., 1920, 182, p. 389.

<sup>8</sup> Some months later, in November, 1920, A. G. suffered a severe attack of acute tonsillitis, with pus crypts and inflammation locally and constitutional symptoms. The house officer of the Barnes Hospital, St. Louis, who made cultures from the tonsils reported a few streptococci, a few staphylococci, but predominantly *M. catarrhalis*. We believe it improbable that this organism was the cause of his symptoms at this time, but it is at least of interest to have found *M. catarrhalis* in this one man, and not in any of the other 4 subjects studied, on 3 different occasions during a 17 month period.

<sup>9</sup> Bull. Johns Hopkins Hosp., 1921, 32, p. 33.

<sup>10</sup> For discussion of the relation of bacteria and of chilling to upper respiratory infection see Jour. Lab. & Clin. Med., 1921, 6, pp. 175, 253 and 322.



# THE ANTIBODIES FOR SHEEP BLOOD AND COMPLEMENT OF THE AQUEOUS HUMOR IN NORMAL AND IMMUNIZED RABBITS

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The antibodies and complement of the aqueous humor in normal and immunized conditions have not been investigated to any considerable extent in systematic manner. As it is possible that a careful study of the aqueous humor from the immunologic point of view might throw light on such problems as the permeability of blood vessels for various immune bodies, the work now to be reported in brief was undertaken. The anterior chamber of the eye with its bloodless and transparent corneal covering would seem to be an ideal place to secure a suitable special fluid for repeated examination by immunologic methods. The few previous investigations of special interest in connection with this work will be considered in the discussion of my results.

## METHODS

Healthy rabbits weighing from 1 to 2½ kilos were used. Blood was obtained in the usual manner from the ear vein under sterile precautions. Aqueous humor was obtained by means of a fine, sterile needle (No. 23) connected with a tube graduated in hundredths of c c; the humor was removed slowly, always without pressure or suction, and with special care to avoid any injury to the iris or other structures. The slightest suspicion that any admixture with blood or plasma had taken place as an immediate result of the puncture eliminated that particular fluid from the tests. The amount obtained in each case varied from 0.2 to 0.27 c c. Usually serum and aqueous humor were obtained in the morning, before the animals were fed, and tested the same day. In any case the serum and fluid were kept in the icebox always until used. To inactivate heating at 56 C. for 30 minutes was practiced. All dilutions were made with sterile 0.9% salt solution. Carefully washed sheep corpuscles were used in the hemolysis tests and fresh guinea-pig serum served as complement unless otherwise stated. Usually the hemolytic mixture consisted of 0.1 c c of serum or aqueous humor, either in full strength or diluted, 0.1 c c of a 5%

suspension of corpuscles, and 0.003 c c of guinea-pig serum, the total quantity being made 0.3 c c by adding salt solution. After incubation for 2 hours the tubes were placed in the icebox and the results recorded the next morning. The hemolytic titer was determined by the highest active dilution of serum or humor which gave a definite trace of positive reaction under careful controls, and frequently several tests were made to determine the accuracy of the results. The tests for agglutinins for red corpuscles were made with heated (56 C.) serum or humor, the quantity being 0.1 c c or less, 5% suspension of well washed corpuscles 0.1 c c, and salt solution to make 0.3 c c, incubated for one hour, the final reading being made the next morning, the tubes in the meantime being kept in the icebox. The precipitin tests were made with fresh sheep serum diluted with salt solution, 0.25 c c of the dilution in each tube, 0.05 c c of the serum or humor being introduced at the bottom so as to secure a sharp line of contact; the results were determined after one hour at room temperature. In the opsonin tests, fresh as well as heated (56 C.) serum or humor was used; 0.1 c c of the serum or humor and of the corresponding dilutions was mixed with 0.1 c c washed sheep corpuscles (5% suspension) and 0.1 c c of pleural exudate, carefully washed and produced by injecting rabbits with aleuronat suspension; sometimes the cream of rabbit blood was used to furnish the leukocytes. The leukocytes were kept always at body temperature to exclude deterioration. The opsonic mixtures were incubated at 37 C. for one hour when smears were stained by Wright's method. From 50 to 150 leukocytes were studied in each case to determine the percentage of phagocytic cells. The opsonic titer was fixed by finding the highest dilution of serum or humor that exercised greater opsonic effect and induced more phagocytes than in the control mixture without serum or humor.

#### ANTIBODIES FOR SHEEP BLOOD IN SERUM AND AQUEOUS HUMOR IN NORMAL RABBITS

Two sets of 3 animals in each were studied and the results are illustrated in tables 1 and 2. The results show that there is a considerable variation in the lysin content of the serum of normal rabbits, the titer running from 96 to 768, and that the titer appears to increase somewhat after a first decrease with repeated bleedings, perhaps as the result of a stimulation of the bloodmaking organs. As to the aqueous humor, it is to be noted that on first puncture it seemed usually not to contain any lysin; occasionally a trace (titer of 3) could be detected.

TABLE 1

TITERS OF HEMOLYTIC AMBOCEPTOR IN SERUM AND AQUEOUS HUMOR OF NORMAL RABBITS

Days Between Bleedings	Titer of Serum			Right or Left Eye	Days Between Punctures	Titer of Aqueous Humor		
	Rabbit 1	Rabbit 2	Rabbit 3			Rabbit 1	Rabbit 2	Rabbit 3
	192	96	768	L	...	0	0	0
2	192	48	768	R	...	0	0	0
2	192	96	384	L	4	0	0	0
2	96	96	192	R	4	0	0	0
2	96	96	192	L	4	3	3	2
3	192	192	384	L	3	3	3	3
5	192	192	384					

Serum heated at 56 C. for 30 minutes; aqueous humor not heated; complement, guinea-pig serum, 0.003 c.c.

TABLE 2

TITERS OF LYTC AMBOCEPTOR, AGGLUTININ AND OPSONIN FOR SHEEP CORPUSCLES IN SERUM AND AQUEOUS HUMOR OF NORMAL RABBITS

Days Between Bleedings and Punctures of Anterior Chambers	Rabbit 1					Rabbit 2					Rabbit 3				
	Serum			Aqueous Humor		Serum			Aqueous Humor		Serum			Aqueous Humor	
	Lysin	Ag- glu- tinin	Op- so- nin	Lysin	Op- so- nin	Lysin	Ag- glu- tinin	Op- so- nin	Lysin	Op- so- nin	Lysin	Ag- glu- tinin	Op- so- nin	Lysin	Op- so- nin
	192	10	....	0	..	96	20	....	0	..	384	20	....	0	..
7	192	10	640	0	20	48	10	320	0	..	384	10	80	3	..
6	96	10	$\frac{640}{5}$	0	$\frac{10}{0}$	48	10	320	3	5	192	20	320	3	5
5	192	10	$\frac{1280}{10}$	0	$\frac{10}{0}$	96	20	640	0	5	1536	40	640	3	10
4	192	10	$\frac{2560}{10}$	0	$\frac{20}{0}$	96	10	640	0	5	1536	20	640	0	10
3	192	10	$\frac{2560}{10}$	0	$\frac{20}{0}$	96	10	320	0	10	1536	20	320	3	10
2	192	10	....	0	$\frac{40}{0}$	96	10	—	0	10	1536	20	....	3	10
1	384	10	$\frac{640}{5}$	0	$\frac{40}{0}$	768	10	1280	0	20	384	20	1280	24	20

Serum and aqueous humor heated to 56 C. for lysin and agglutinin tests, but not for opsonin tests. Figures under the line under opsonin, Rabbit 1, given opsonin titer of heated serum and humor. No agglutinin for sheep corpuscles in aqueous humor. No trace of precipitin for sheep serum in either serum or humor. Both chambers punctured at same time.

The relative content of lytic amboceptor in aqueous humor and blood stands in an approximate ratio of about 1 to 256. With repeated puncture at intervals of over two days a small amount of lytic amboceptor occasionally appears in the humor. In the course of the experiments it was observed that after removal of the aqueous humor the content of the anterior chamber may return to the normal condition usually within 24 hours. In some special experiments punctures of the anterior chamber were made at intervals of 30 minutes and, as shown in table 3, the amount of lysin in the fluid withdrawn on the second puncture was vastly greater than that in the fluid of the first, the titers in the native humors running from 24 to 96 or about one-fourth to one-eighth the strength of the corresponding serum. This result shows how easily the blood vessels permit the passage of lysin under conditions of changed pressure without any inflammatory or other changes. It is noteworthy

TABLE 3  
CONTENT OF HEMOLYTIC AMBOCEPTOR FOR SHEEP CORPUSCLES IN AQUEOUS HUMOR ON  
TWO PUNCTURES, 30 MIN. APART

Punctures	Rabbit 1	Rabbit 2	Rabbit 3	Remarks
First.....	3	3	3	
30 minutes later	48	24	96	Aqueous humor heated at 56 C. for 30 minutes; complement guinea-pig serum, 0.003 c c

that, as already indicated, in about 24 hours the normal conditions of the aqueous humor are restored fully as a rule.

The normal rabbit serum unheated causes lysis of sheep corpuscles in dilutions up to 1:24, but the aqueous humor in full strength, that is, one part of fluid to one part of 5% suspension of corpuscles, is without any lytic effect.

Next to be considered was the amount of complement in normal rabbit serum, whether any complement occurs in the normal aqueous humor, and whether complement enters the aqueous humor on puncture of the anterior chamber.

Using the serum of a rabbit that was found to contain an average amount of amboceptor titer 192, I tried to complement this amboceptor with the aqueous humor of several normal rabbits, but in no case did the humor obtained on the first puncture in the quantity of 1 c c serve to complement the amboceptor. One example is shown in table 4. On second puncture, 30 minutes later, however, the humor was found to contain some complement that clearly must have passed into the anterior

chamber from the blood as a result of the changes in pressure. Compared with the amount of complement in the serum, the amount in the aqueous humor obtained on second puncture was on an average about one-fourth the concentration of the complement of the serum. Here I may point out that the amount of complement in normal rabbit serum varies greatly, being always less than in guinea-pig serum, from  $\frac{1}{8}$  to  $\frac{1}{4}$  as much. There is no parallelism between the amount of complement and amboceptor in the normal rabbit.

As shown in table 2, the agglutinin titer of normal rabbit serum for sheep corpuscles is between 10-20, occasionally being as high as 40; in the aqueous humor, however, no agglutinin was found in any case on the first puncture.

TABLE 4  
REACTIVATION OF RABBIT SERUM HEATED TO 60 C. FOR 10 MINUTES WITH NORMAL RABBIT SERUM AND ORIGINAL AQUEOUS HUMOR AND AQUEOUS HUMOR DRAWN 30 MINUTES AFTER FIRST PUNCTURE

Rabbit Serum Heated at 60 C. for 10 Minutes, C c	5% Suspension of Sheep Corpuscles, C c	Fresh Rabbit or Guinea-Pig Serum or Aqueous Humor, C c	Hemolysis			
			Fresh Rabbit Aqueous Humor		Fresh Rabbit Serum	Fresh Guinea-Pig Serum
			First Puncture	Second Puncture		
0.1	0.1	0.1	0	+++	++++	++++
0.1	0.1	0.05	0	++	++++	++++
0.1	0.1	0.025	0	+	+++	++++
0.1	0.1	0.0125	0	0	+	+++
0.1	0.1	0.00625	0	0	±	++
0.1	0.1	0.003	0	0	0	+
—	0.1	0.1	0	0	0	0
0.1	0.1	—	0	0	0	0

Total amount made 0.3 c c by adding normal salt solution.

The titer of the opsonin of fresh normal rabbit serum for sheep corpuscles was found to run from 80-640, and that of normal aqueous humor of the same rabbits to vary from 5-20. After heating the serum, the titer was found to be about 5 and after heating the aqueous humor no opsonic effects could be demonstrated.

On repeated puncture of the anterior chamber, the titer of the thermolabile opsonin of the aqueous humor rose occasionally to 40, but it is to be emphasized that probably as the result of repeated bleedings during this period, the titer of the serum of the rabbits had increased to 2,560. While the thermostable opsonic substance in the normal serum had risen to a titer of 10, the aqueous humor still remained free from any demonstrable such opsonin. In no case was any trace of precipitin for sheep proteins found in either the serum or the aqueous humor of normal rabbits.

RELATIVE CONCENTRATION OF HEMOLYSIN AND OTHER ANTI-  
BODIES IN SERUM AND AQUEOUS HUMOR OF RABBITS  
IMMUNIZED WITH SHEEP BLOOD

As stated, the titer is represented by the highest dilution of serum or humor that gave a definite trace of lysis. The animals received 30 c.c. of defibrinated sheep blood on one injection and were subsequently bled every 2 or 3 days, while the anterior chamber was punctured with the greatest care at intervals of at least 2 days. The lysin curves of the serum of the animals were similar in outline but varied in height, one rabbit giving a very high curve and 2 others moderately

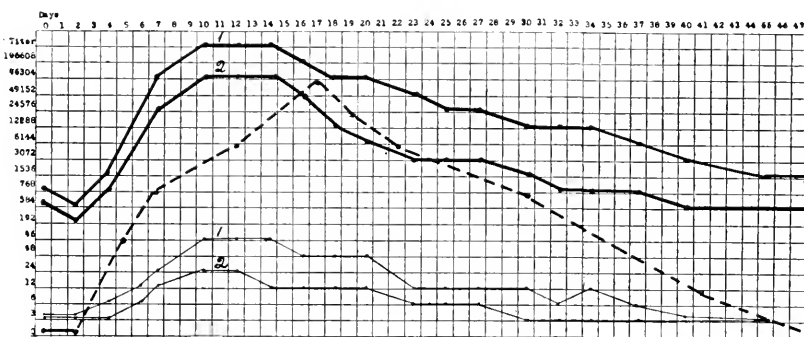
TABLE 5  
HEMOLYTIC AMBOCEPTOR IN SERUM AND AQUEOUS HUMOR OF RABBITS INJECTED INTRA-  
PERITONEALLY WITH 30 C.C. OF SHEEP'S BLOOD

Days After Injection	Rabbit 1		Rabbit 2*		Rabbit 3	
	Serum	Aqueous Humor	Serum	Aqueous Humor	Serum	Aqueous Humor
Before (2 days)	768	3	384	3	384	3
2	384	3	384	3	192	3
4	1,536	6	3,072	3	768	12
6	24,576	12	98,304	6	6,144	24
7	98,304	24	78,642	48	24,576	24
10	393,216	96	.....	768	98,304	24
12	393,216	96	.....	..	98,304	24
14	393,216	96	.....	1536	98,304	12
16	196,608	48	.....	..	49,152	12
18	98,304	48	.....	..	12,288	12
20	98,304	48	786,432	24	6,144	12
23	49,153	12	398,216	12	3,072	6
25	24,576	12	196,608	12	3,072	6
27	24,576	12	98,304	12	3,072	6
30	12,288	12	24,576	12	1,536	3
32	12,288	6	12,288	6	768	3
34	12,288	12	12,288	12	768	3
37	6,144	6	6,144	6	768	3
40	3,072	6	3,072	6	384	3
45	1,536	3	Died	..	384	3
51	768	3	.....	..	384	3

\* This rabbit produced an enormous amount of amboceptor, the titer going into the millions at the height. In the meantime the animal became extremely weak and thin so that bleeding and punctures had to be omitted at times.

high curves. The 2 latter presented a negative phase for 2 days after the injection. In all cases the lysin production set in on the 4th day and rose steadily, reaching the highest point on the 10th day except in the animal that gave the highest curve; in this the apex was reached on the 14th day. In all the animals the descent of the curve began on the 14th day and reached the normal in one case on the 40th day, in another the titer was still a little higher than normal on the 51st, while the rabbit with the very high curve died on the 40th day without any other lesions except a progressive emaciation.

In the aqueous humor of these rabbits there was a definite rise in lysin, running parallel with the lysin in the blood serum, but very much lower and somewhat less regular. The increase began on the 4th day in 2 animals and on the 6th in 1, reached the high point on the 10th or 12th day when a gradual fall began, the normal level being reached on the 30th-40th day. As shown in table 5, the hemolysin titer of the humor reached from 24 to 96, and as high as 1,536 in an extraordinary case; the ratios of the titer of the aqueous humor of these animals to the titers of the serum at the highest points of curves were 1:4,096 in two of the animals and 1:16,384 in the third. At the time when the titer of the humor began to rise, the titer of the serum was from 756-1,536. We see that the relation between the titer of the aqueous humor and blood serum now is not the same as in the normal animal.



Hemolysin curves of blood serum (heavy solid lines) and of aqueous humor (fine lines) of Rabbits 1 and 2 each injected intraperitoneally with 30 c.c. of sheep's blood and precipitin curve (broken line) of serum of rabbit injected with 40 c.c. of sheep's blood intraperitoneally; no precipitin in the aqueous humor of this rabbit

Both precipitin and agglutinin appeared in the serum on the fifth day after the injection of 40 c.c. of sheep's blood in another set of rabbits (table 6) and increased in amount steadily, the precipitin reaching its height on the 16th day and the agglutinin on the 12th; in both cases a gradual fall set in; on the 16th day and on about the 50th day all evidence of these antibodies had disappeared from the blood. The aqueous humor of the same rabbits failed to show any trace of precipitin or agglutinin. It would seem as if there is some hindrance to the passage of these antibodies into the anterior chamber, possibly because of the size of their molecules.

The intraperitoneal injection of 40 c.c. of defibrinated sheep blood into rabbits was followed by a rise in the opsonic power of the fresh

serum for sheep corpuscles beginning about the 5th day and reaching its highest point on the 16th day; on the 22nd day a fall began, the normal level being reached again on about the 49th day. There was a parallel rise in the opsonic power of the fresh (unheated) aqueous humor, the relation of the titer to that of the serum at the height of the curve being 160:20,480 or 1:128.

The hemolytic power of the fresh serum of rabbits injected with sheep's blood showed only a slight increase when tested without the addition of complement of other source, and the aqueous humor under these circumstances was without any lytic effect. It is clear from this result that while there is a marked increase in amboceptor after the injection of sheep's blood, there is no apparent change or increase in the native complement of the rabbit serum.

TABLE 6  
OPSONIN, AGGLUTININ AND PRECIPITIN IN SERUM AND AQUEOUS HUMOR OF RABBITS  
INJECTED INTRAPERITONEALLY WITH 40 CC OF SHEEP'S BLOOD

Days After Injection	Opsonin				Agglutinin in Serum	Precipitin in Serum
	Serum		Aqueous Humor			
	Unheated	Heated	Unheated	Heated		
	320	5	20	0	5	0
2	—	—	—	—	5	0
5	640	..	20	0	20	80
8	1,280	—	40	0	40	640
12	4,120	160	80	0	80	5,120
16	20,480	640	160	0	80	81,920
19	20,480	640	160	0	40	20,480
22	20,480	160	160	0	40	5,120
26	10,240	320	80	0		
30	2,560	80	40	0	40	640
35	1,280	20	40	0	20	
42	640	10	20	0	10	10
49	320	5	20	0	5	0

No agglutinin or precipitin in aqueous humor.

It appears that in the course of immunization with sheep blood the only antibodies to appear anew in the aqueous humor are lysin and opsonin.

#### DISCUSSION

According to Gatti,<sup>1</sup> the aqueous humor in normal cattle does not contain any hemolysin, but in 5 of 13 dogs this humor was lytic for rabbit corpuscles, according to Hughes and Carlson.<sup>2</sup> Becht and Greer,<sup>3</sup> however, found no lysin in the aqueous humor of 10 normal

<sup>1</sup> Biochem. Centrbl., 1905, 4, p. 678.

<sup>2</sup> Am. Jour. Physiol., 1908, 21, p. 245.

<sup>3</sup> Jour. Infect. Dis., 1910, 7, p. 131.



dogs, and they say that even dogs injected with rabbit's blood do not show any lysin or agglutinin in the aqueous humor. According to my observation, the aqueous humor of normal rabbits sometimes may contain a trace of lysin for sheep corpuscles. Generally speaking, it would seem that depending on individual variation the hemolytic titer of the aqueous humor of normal rabbits runs from 0-3. This variation may be related to the normal hemolytic titer of the serum which I found may vary between 96 and 768, repeated bleedings tending to increase the titer. I was not able to demonstrate any hemagglutinin in the normal rabbit aqueous humor, and the same is also true of precipitin for sheep serum; opsonin for sheep corpuscles, however, was found to be present in small quantities.

On active immunization with sheep blood, the aqueous humor, under otherwise normal conditions, may contain lytic amboceptor in noticeably greater amount than normally. In no case did any hemagglutinin or precipitin appear in the aqueous humor after the injection of sheep blood. These observations are in full harmony with the results obtained by Hektoen and Carlson,<sup>4</sup> who found that after the injection of dogs with goat blood new lysin appeared in the aqueous humor but no agglutinin. But Wessely<sup>5</sup> makes the general statement that agglutinin and hemolysin exist only in a minimal amount in the intra-ocular fluid of the most highly immunized animals.

Metchnikoff<sup>6</sup> and Mesnil<sup>7</sup> noted that alexin was absent from the aqueous humor, and Metchnikoff believed that this was due to the fact that there are no leukocytes in this fluid. In my work, complement was never found in the aqueous humor of normal rabbits. Roemer,<sup>8</sup> Falloise,<sup>9</sup> Sweet,<sup>10</sup> and Miyashita<sup>11</sup> report similar observations. It is of interest to note that as a rule the pericardial and cerebrospinal fluids do not contain complement.

After immunization with sheep blood, specific precipitin for sheep serum develops in large quantities in the serum, reaching in one of my animals a titer of 81,920, but in no case was any trace of precipitin demonstrable in the aqueous humor. Von Dungern<sup>12</sup> also found that

<sup>4</sup> *Ibid.*, 1, p. 319.

<sup>5</sup> *Klin. Monatsbl. f. Augenheilk.*, 1902, 40, p. 267.

<sup>6</sup> *Ann. de l'Inst. Pasteur*, 1895, 9, p. 438.

<sup>7</sup> *Ibid.*, 1896, 10, p. 369.

<sup>8</sup> *Arch. f. Augenheilk.*, 1906, 54, p. 207.

<sup>9</sup> *Bull. de l'Acad. Roy. Belg.*, 1905, 5.

<sup>10</sup> *Zentralbl. f. Bakteriolog.*, 1903, 33, p. 208.

<sup>11</sup> *Klin. Monatsbl. f. Augenheilk.*, 1910, 48, p. 293.

<sup>12</sup> *Die Antikörper*, Jena, 1903, p. 113.

the aqueous humor of animals injected with the plasma of *Maja aquinada* was free from precipitin while the serum contained large quantities, and would remain free unless the iris became injured in some way. Becht and Greer<sup>3</sup> also found no precipitin in the aqueous humor of a dog injected with a large amount of rabbit blood.

On immunization new opsonin may make its way into the aqueous humor in rabbits just as Hektoen and Carlson<sup>4</sup> found it to do in dogs injected with goat blood. As hemolytic amboceptor may exist in the aqueous humor of rabbits without the presence of any complement, the humor nevertheless containing opsonin, it would seem that it concerns different substances.

In the course of my experiments the anterior chamber was punctured frequently at intervals, beginning 2 days after the injection, up to several days in animals injected with sheep blood, hence sheep protein might probably enter the aqueous humor. If antibodies can be produced locally, one would expect especially that there would be an accumulation in the anterior chamber, but such did not seem to be the case as the curve of the lysin was exactly like that of the serum only much lower, and no hemagglutinin or precipitin seemed to arise locally. My results seem to support the conclusions of Hektoen<sup>13</sup> that antibodies may enter the aqueous humor from the blood and lymph and not as the result of any local production.

#### CONCLUSIONS

The serum of normal rabbits contains lysin and opsonin, also agglutinin in small quantities for sheep corpuscles, but no precipitin for sheep serum.

The aqueous humor of normal rabbits contains a small amount of opsonin and lysin for sheep corpuscles, but no agglutinin or complement and no precipitin for sheep serum.

On immunization with sheep blood the antibodies mentioned accumulate in the blood, but the aqueous humor gives an increase in lysin and opsonin only, remaining free from the other antibodies.

On removal of aqueous humor by puncture, the new fluid that forms has a far greater amount of antibodies in it than the original fluid. The normal conditions are regained in about 24 hours.

<sup>13</sup> Jour. Infect. Dis., 1911, 9, p. 103.

## FOOD ACCESSORY FACTORS IN BACTERIAL GROWTH

### III. FURTHER OBSERVATIONS ON THE GROWTH OF PFEIFFER'S BACILLUS (B. INFLUENZAE)

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In a previous article,<sup>1</sup> I called attention to the rôle of two substances in the growth of Pfeiffer's bacillus; one, hemoglobin or a derivative and the other, a substance obtainable from plant tissues (carrot, potato), animal tissue, bacteria, yeasts, etc. There are two methods by which these processes have been studied—one, by adding the substances in question to plain blood agar or broth in test tubes and observing the growth after inoculation with Pfeiffer's bacillus; the other by making a poured blood unheated or heated) agar plate, seeding heavily with Pfeiffer's bacilli and then inoculating here and there with bacteria, yeasts, pieces of tissues, etc. About the latter, Pfeiffer's bacilli will develop large or "giant" colonies, in this manner forming a cluster of colonies about the central foreign colony or tissue and known as the "satellite" phenomenon.<sup>2</sup> Further experiments have been made as to the mechanism and the nature of the substances involved in the growth processes of this organism which I wish to report now.

In the following experiments at least two strains of Pfeiffer's bacilli were used; one isolated from a pneumonic lung during the 1919 epidemic, the other from the spinal fluid of a case of so-called influenzal meningitis in a child. No differences of behavior were noticed between these organisms. In certain experiments other respiratory strains of Pfeiffer's bacilli were used with comparable results.

Blood mediums when moderately heated will give a profuse growth of Pfeiffer's bacillus; when heated in the autoclave for 30 minutes it yields practically no growth. The autoclaved medium now may be made to yield excellent growth by adding thereto certain substances which in themselves do not allow growth when added to plain medium.

Received for publication April 18, 1921.

<sup>1</sup> Jour. Infect. Dis., 1917, 21, p. 392.

<sup>2</sup> Davis, D. J., Ibid, p. 178.

Such substances include tissues of various kinds, or their extracts or filtrates, such as carrot, potato, and animal tissues. However, when these tissues or their extracts are heated to the boiling point for a time (1 to 2 hours) or are autoclaved they will no longer activate the autoclaved blood. Table 1 illustrates these points.

TABLE 1  
GROWTH OF PFEIFFER'S BACILLUS

Plain medium + autoclaved blood .....	=	0
Plain medium + carrot or potato filtrate .....	=	0
Plain medium + autoclaved blood + carrot or potato filtrate .....	=	+++
Plain medium + autoclaved blood + autoclaved filtrate .....	=	0
Plain medium + washed heart muscle (guinea-pig) .....	=	0
Plain medium + autoclaved blood + heart muscle .....	=	+++
Plain medium + autoclaved + autoclaved heart muscle .....	=	0

Various plant tissues may be used in this experiment and also various animal tissues, such as liver, heart muscle, kidney, brain, spleen, etc. There is an advantage in using plant tissues because they contain no hemoglobin. However, in using animal tissues for activating purposes, by washing small pieces for a long time one can remove this substance. Furthermore by employing the satellite test one can observe the activating effect even on medium containing unheated hemoglobin, so there is no doubt but that animal tissues behave in the same way as plant tissues.

In addition to the plant and animal tissues, various lower organisms may be used in the same manner, either in the form of suspensions or as extracts or filtrates (Berkefeld). Table 2 shows this fact.

TABLE 2  
GROWTH OF PFEIFFER'S BACILLUS

Plain medium + autoclaved blood .....	=	0
Plain medium + bacteria .....	=	0
Plain medium + autoclaved blood + B. coli .....	=	+++
Plain medium + autoclaved blood + B. coli filtrate .....	=	+++
Plain medium + autoclaved blood + heated B. coli (60 C.-30 m.) .....	=	+++
Plain medium + autoclaved blood + heated B. coli (100 C.-5 min.) .....	=	+++
Plain medium + autoclaved blood + heated B. coli (autoclaved) (30 min.) ..	=	0

In this experiment other organisms, like staphylococci, streptococci, sporotricha, blastomycetes, yeasts, etc., may be used, little difference being noted, provided, of course, the reaction is not appreciably altered or is properly adjusted. It will be seen that here again bacteria and other organisms or their filtrates activate the autoclaved blood medium.

By heating the organisms this activating power is gradually reduced so that exposure to the temperature of the autoclave for 30 minutes will cause it to disappear entirely.

It seems clear that we are concerned with two substances, and we may now in the light of these experiments examine further into the behavior of Pfeiffer's bacillus grown on blood and serum medium. Table 3 reveals the facts in condensed form.

TABLE 3  
GROWTH OF PFEIFFER'S BACILLUS

Plain medium + crystallized hemoglobin .....	=	+	(slight)
Plain medium + unheated blood .....	=	+	(slight)
Plain medium + blood (heated to 60 C. for 5 hours) .....	=	+++	
Plain medium + blood (heated to 100 C. for 5 minutes) .....	=	+++	
Plain medium + blood (autoclaved 30 minutes).....	=	0	
Plain medium + autoclaved blood + serum .....	=	+++	
Plain medium + autoclaved blood + autoclaved serum .....	=	0	
Plain medium + serum .....	=	0	

From this experiment it is seen that crystallized hemoglobin or ordinary fresh blood plus plain medium is not a good medium though definite growth will occur. When corpuscles are heated to a certain point, however, their value to a medium is markedly enhanced. When heated beyond this point their value is destroyed.

Serum, when added to autoclaved blood medium, will reactivate it promptly, yielding a medium very favorable for growth. Pure serum alone added to plain medium does not yield a growth. Ascitic fluid, if fresh and of high specific gravity, behaves like serum but when of low specific gravity or old it has little or no action. When heated to boiling for 2 hours or autoclaved for 30 minutes the reactivating power of serum and ascites fluid is destroyed. Evidently, then, the serum and the ascites fluid behave quite like plant and animal tissues and also like bacteria and their filtrates referred to.

When ordinary unheated blood (defibrinated or whole blood) is added to plain medium the growth though definite is not abundant. When heated to 55 C. even indefinitely growth also is slight or at times absent. At 60 C. growth is not profuse unless this temperature is applied from 2 to 5 hours. If continued for 2 to 3 days no growth results. At 100 C. a few moments' exposure or simply bringing the medium to this temperature is sufficient to allow profuse growth and exposure for 1 to 2 hours destroys its growth producing value. At 120 C. (autoclave) a few minutes' exposure of the blood mediums

renders it valueless. Thus with increasing temperature the time necessary to obtain a favorable medium becomes less and less and also with increasing temperature the time necessary to destroy its growth value becomes gradually less.

These facts are interpreted as meaning two things. In the first place, the hemoglobin in itself is not a good medium for Pfeiffer's bacillus, perhaps will not support growth at all, and only when it has been changed by heat to hematin or some closely related derivative can it cooperate with a second substance in the blood. This change of hemoglobin appears to take place slowly at 60 C. but more rapidly at higher temperatures. This amount of heat as shown, however, does not destroy the second substance in the blood or the serum; therefore we have the two substances within certain ranges of heating operating together and yielding a profuse growth. If the heating is continued, the second substance is destroyed and no growth takes place without reactivation.

A medium in the form of albumin or peptone appears to be necessary for, as I have shown in previous papers, hemoglobin or hematin alone does not support growth of this organism. The heat resistant substance appears to be hematin or hemin since the action of heat on hemoglobin results in the formation of these substances.

One other point should be mentioned. At or even below about 55 C. blood is coagulated and becomes chocolate in color. However, such blood medium, even though heated for many days (3 weeks), does not yield a favorable medium. When activated with sterile carrot juice the growth is profuse. Blood medium heated at 60 C. or above for 2 or 3 hours yields a good growth without the addition of carrot or tissue juice. My interpretation of this fact is that the temperature of 55 C. or thereabouts is not sufficient in a certain time to cause the change in the hemoglobin resulting in the formation of the derivatives necessary to maximum growth. On the other hand, this temperature continued long enough renders inactive the second substance. There is therefore this interval in the heating of blood in which a profuse growth does not result. Beyond this temperature the hemoglobin is rapidly changed, and heating at the boiling point or at autoclave temperature for hours does not destroy the heat resistant substance (hematin) formed.

As to the second substance: Its heat relation has already been presented. It readily passes through Berkefeld filters and appears to be a product intimately related to living cells. The question as to the

possible relation of this body to growth factors or vitamins naturally arises here. It has been discussed by me in a previous paper<sup>1</sup> and, as then pointed out, there are features about it that suggest that we are dealing with a body of that nature. However, so little is known of the real character of these substances and the criteria for their identification are so indefinite, that little more can be done now to raise the question. I think that both of these bodies may be spoken of as growth or food accessory factors for this organism, using the term in the sense that growth processes depend on them. Whether the mechanism here involved is the same as the mechanism of vitamins in animal growth is not known. However, through the study of such bodies which, as I have detailed, are found in bacteria, yeasts and other tissues, we may be able to throw light on the real mechanism by which vitamins operate. Possibly the ultimate source of these substances may be found in the realm of these lower bacterial organisms. As pointed out,<sup>1</sup> this phenomenon, so far as the growth of Pfeiffer's bacillus is concerned, would seem to center about the metabolism of iron, and this would suggest that the processes are in the main concerned with oxygen or its transfer.

Other agents appear to be able to alter hemoglobin in the same way as heat. The decomposition or digestion of blood by bacteria of various kinds, if not prolonged, yields a product which gives abundant growth. This may be shown by adding putrified blood filtrate to medium in a test tube and inoculating with Pfeiffer's bacilli. If the decomposition of the blood has gone on for a long time (2 to 3 weeks by *B. coli* for example) no growth of Pfeiffer's bacillus will result on medium to which it has been added. Such medium can be reactivated, however, by adding to it fresh carrot or potato juice or fresh unheated blood, serum or animal tissue. The so-called peptic digest used by Fildes<sup>3</sup> no doubt contains the same substances, the pigment portion being reactivated by the supernatant fluid.

It was shown many years ago by Ghon and Preyss<sup>4</sup> that pure hematin alone would not support the growth of this bacillus but when used on hematin medium with another organism, good growth would result. This has been confirmed by others, including myself, and is in entire accord with the observations detailed now on heated hemoglobin, assuming that hematin results during this process. Hemin also

<sup>3</sup> Brit. Jour. Exper. Path., 1921, 2, p. 16.

<sup>4</sup> Centralbl. f. Bacteriol., 1902, 32, p. 96.

is stated by Olsen<sup>5</sup> to support growth along with other organisms and this substance, too, may result during the process of heating. I have not tested this point myself.

I have attempted to activate many iron and other compounds with substances like carrot juice and bacteria, but I have not been able consistently to do so. The iron derivatives of hemoglobin appear to be the only ones that will so react. I have gone into this point in considerable detail in another paper,<sup>2</sup> and I shall discuss it here no further than to state that for this purpose tests may be readily, and I think delicately made by the plate method used for determination of satellitism.

The relation of this process to the guaiac reaction has been discussed recently by Olsen,<sup>5</sup> who points out that a parallelism exists between the ability of Pfeiffer's bacillus on medium containing hemoglobin and its derivatives and a positive guaiac test. A derivative not containing iron, like hematoporphyrin, will give neither. He does not discuss the question of the reactivation of heated blood by different substances. Fildes<sup>3</sup> also discusses this question but does not explain the fact that many iron and other compounds give a positive guaiac test but do not promote the growth of Pfeiffer's bacillus. He also raises the question as to the possibility of the second substance being a peroxide of such a nature that through the catalytic action of hematin the transfer of oxygen to the bacillus from the peroxide is accelerated. He was led thus to the conclusion to which I was led some years ago<sup>4</sup> through a study of the behavior of blood pigments in high dilutions, namely, that the nature of this process is catalytic.

#### SUMMARY

Pfeiffer's bacillus grows feebly on mediums containing unheated blood.

Blood mediums heated to 60 C. or higher for definite periods of time yield profuse growth of the bacillus.

Heating in the autoclave (120 C.) for a few minutes or at lower temperatures for longer periods renders blood medium incapable of growing Pfeiffer's bacillus.

This superheated blood medium may be reactivated by adding to it plant, animal and bacterial extracts and filtrates which by them-

<sup>5</sup> Ibid., 1920, 85, p. 12.

<sup>6</sup> Davis, D. J.: Jour. Infect. Dis., 1907, 4, p. 73.



selves do not support growth of this organism. The latter substances lose this property on heating at autoclave temperature for a few minutes or at a lower temperature for longer periods.

The growth process of Pfeiffer's bacillus may be represented thus :  
plain medium + heat resistant substance (hematin or derivative) + heat labile substance = growth of Pfeiffer's bacillus.

# THE ACCESSORY FACTORS IN BACTERIAL GROWTH

## IV. THE "SATELLITE" OR SYMBIOSIS PHENOMENON OF PFEIFFER'S BACILLUS (*B. INFLUENZAE*)

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If one makes an ordinary blood-agar plate by pouring a tube heavily inoculated with Pfeiffer's bacillus (*B. influenzae*), colonies will appear which are very tiny; often apparently many of the organisms do not grow at all. However, if some other organism, a staphylococcus for example, is inoculated onto the plate and the plate incubated for 24 hours, the Pfeiffer colonies close to the staphylococcus colonies will appear very much larger than those in other parts of the plate and also apparently more numerous. Thus, there appears a prominent central colony (staphylococcus) surrounded by a cluster of relatively large Pfeiffer colonies. This has been termed the "satellite" phenomenon.

In 1897, Grassberger<sup>1</sup> first pointed out that close to the margins of colonies of *Staph. aureus* the Pfeiffer bacillus tended to form giant colonies. He also showed that if the staphylococcus cultures were killed by heat ( $\frac{1}{4}$  hour duration) and mixed with blood medium, the growth of the Pfeiffer bacillus was markedly enhanced. He therefore concluded that the bacterial products and not the symbiosis favorably influenced the growth of Pfeiffer's bacillus. In 1898, Meunier<sup>2</sup> noted the relatively large colonies of Pfeiffer's bacillus about other colonies grown in plate cultures with it, and referred to the phenomenon as "cultural satellitisme." Allen<sup>3</sup> observed it on plates when isolating bacteria from certain cases of chronic influenza. He grew Pfeiffer bacilli on medium to which heat killed staphylococcus albus had been added and noted a growth far more profuse than on ordinary blood mediums, attributing it to a toxin highly adjuvant to the growth of Pfeiffer's bacillus. He made identical experiments with *Pneumococcus*, *Staph. aureus*, *B. coli*, *B. acidi-lactici* and *Microc. paratetrigenus*, all of which behaved alike.

The favoring action of organisms on the Pfeiffer bacillus has been observed and noted by many other observers. For years it has been customary to smear the entire slant surface of blood agar with Pfeiffer's bacilli and then make a light streak with staphylococcus

Received for publication April 18, 1921.

<sup>1</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1897, 25, p. 453.

<sup>2</sup> Semaine méd., 1898, 18, p. 268.

<sup>3</sup> Lancet, 1910, 1, p. 1263.

through the center in order to enhance the growth of the former. The favoring action is also evident when plating out material, such as sputum, containing a mixed growth of Pfeiffer's bacillus and other organisms, especially if the former are numerous, in which case the cluster of large Pfeiffer's bacilli colonies about colonies of the other bacteria offer a striking appearance.

Further than to note the phenomenon, little has been done to analyze the factors concerned in its production. For this reason, I have thought the results of certain observations, made to determine its nature, worthy of presentation.

No organism other than Pfeiffer's bacillus is known that behaves in this way when grown with other organisms. At any rate, this statement is true when organisms are tested on blood or hemoglobin plates as described. A somewhat similar appearance is presented at times by certain motile bacteria on plates. A large central colony may become surrounded by a cluster of smaller and younger colonies which have arisen from bacteria moving out from the central colony. This phenomenon can easily be differentiated from the satellitism of Pfeiffer's bacillus because the central colony and those clustered about it are the same organism. Since the Pfeiffer bacillus is unique in this regard the phenomenon has become a most valuable means of differentiating it from other closely related bacteria.

The reaction does not appear to be a mutual one. That is, while the Pfeiffer bacilli are favorably influenced in their growth, the central or foreign colony is not affected either favorably or unfavorably. This may readily be shown by planting a portion of a plate with Pfeiffer bacilli and then inoculating with other bacteria. No difference is noted in the size of the latter colonies in the two halves of the plate. Owing to this fact, the question arises as to whether the term symbiosis should be applied to this phenomenon. Since it is not a mutual process it would seem that the term commensalism would be the proper one to use.

While the favoring action of an organism on the growth of Pfeiffer bacilli on solid blood medium is thus definitely manifested, the same action is not so clear on blood medium when the organisms are intimately mixed and transferred in this condition. Here Pfeiffer's bacilli multiply along with certain feebly growing cocci or bacilli (streptococci, diphtheroids) through several generations, gradually becoming less and less numerous. With *Staph. aureus* it dies out in two or three generations. There would appear to be in mixed culture

another factor inhibiting in character and which quite rapidly overcomes Pfeiffer's bacillus. So, too, in fluid blood cultures Pfeiffer's bacillus may grow through a few generations with a streptococcus or a staphylococcus, soon, however, becoming extinct. Further evidence of an inhibition or antagonistic action of bacteria on Pfeiffer's bacillus will be referred to later. In this connection, too, may be mentioned the fact that on nonhemoglobin medium Pfeiffer's bacillus will live through many generations when growing with another organism. I have grown it mixed with a diptheroid through 7 generations on plain mediums which without this bacillus would not grow at all. Neisser grew it through 20 generations with the *B. xerosis* on plain mediums and others have confirmed these results. The question always enters here as to the possible influence that blood in the medium may have since we now know that heated blood plus bacteria will yield a growth. It would appear, then, that a favorable as well as an unfavorable effect is exerted, by staphylococci at least, and also presumably by other organisms.

The zone of favorable influence about a foreign colony varies considerably in width even for one and the same organism. It may be as narrow as 1 mm. or may be 1 cm. or more in width. It is usually clearly visible for a distance of 2 to 3 mm. There is little difference apparently between the width of the zone of influence about different bacteria. It seems wider when the number of colonies is few.

The zone is symmetrical and most intense usually at or near the margin of the central colony. There are, however, certain exceptions to this. Often about staphylococcus colonies one sees a narrow zone of inhibition which may be a fraction of a millimeter in width. Beyond this the colonies of Pfeiffer bacilli are large and prominent. In this narrow zone the colonies are small or entirely absent. About certain other bacterial colonies, too, zones of inhibition appear which may be very wide. I have a strain of *B. subtilis* which is strongly hemolytic and about which there is a zone of inhibition so wide that it apparently neutralizes the favorable influence that might be exerted by the colony. At any rate, that is the interpretation I have given the observation. The zone of hemolysis is nearly 1 cm. in width; the medium is there alkaline and colonies composed of bacteria other than Pfeiffer bacilli (*Staph. albus*, for instance) are also here inhibited. This is the only organism I have met with thus far that does not reveal a favorable action on the Pfeiffer bacillus; though probably others could be found if systematic tests were made with a great number of bacteria.

When using for central colonies certain bacteria that tend to form large spreading growths, one may observe that the adjacent Pfeiffer colonies may be covered by the spreading margins of the central colony. Hence, one might conclude on superficial examination that such a colony does not act favorably on Pfeiffer's bacillus. However, by more careful inspection one may see the enlarged colonies of Pfeiffer bacilli underneath, or apparently in, the large central colony, showing clearly that a satellite zone was early formed and had later been covered or overgrown by the spreading colony. Strains of *sarcina lutea* and *B. mucosus* will at times behave in this manner.

With the exception of these instances, I have not found an organism that will not enhance the growth of Pfeiffer's bacillus. I have examined the following with positive results: *Staph. albus* and *aureus*, *Strept. hemolyticus* and *viridans*, *Pneumococcus* types 1, 2 and 3, also many strains of group 4, *Meningococcus*, *Micrococcus catarrhalis*, *B. diphtheriae*, *B. pseudodiphtheriae* (many strains of diphtheroids), *Sarcina lutea*, *B. coli*, *B. typhosus*, *B. paratyphosus* (A and B) *B. dysenteriae*, *Sp. metchnikovii*, *gonococcus*, *B. mucosus* (Friedländer), *B. pyocyaneus*, *B. prodigiosus*, *B. sporogenes*, *B. enteritidis*, *B. fecalis-alkaligenes*. Fifteen different organisms were isolated on plates from the air. These were chiefly chromogenic organisms and were not definitely identified. All without exception favorably influenced the Pfeiffer bacilli. I also tested on plates many sputum bacteria, and all colonies that grew revealed the satellite phenomenon with the exception of the strain of *B. subtilis* mentioned. No differences were noted between pathogens and saprophytes.

Strains of yeasts stimulate the growth of Pfeiffer's bacillus very well, provided the strain grows appreciably in 24 to 48 hours. With slow growing organisms in general, the phenomenon is not observed because during the short growth period of Pfeiffer's bacillus the former have not had time to multiply sufficiently to exercise any appreciable influence. This is true of tubercle bacilli, *blastomyces*, *sporotricha* and *achorion quinckeanum*. The tests were all made using at least two strains of Pfeiffer's bacillus, one from a case of influenzal meningitis and the other from a case of bronchopneumonia. In some instances many more strains of Pfeiffer's bacillus were used in the tests.

Pfeiffer bacilli are influenced favorably about to the same degree by both hemolytic and nonhemolytic bacteria. However, there is an advantage in using as a central colony one that causes hemolysis like *Strep. hemolyticus* or a hemolytic colon bacillus because in the clear

zone of hemolysis the Pfeiffer colonies can be readily seen, and their variation in size easily ascertained. Furthermore, the phenomenon is equally well brought out by using blood corpuscles hemolyzed by such agents as water. Satellitism, then, is not dependent on, or due to, hemolysis or to liberation of the hemoglobin from the corpuscles because the phenomenon is observed equally well in blood, unlaked or laked by bacteria or by water.

The reaction is noted on plates about both alkali and acid formers. At least, this is true unless large amounts of these substances are generated. The instance cited of a strain of *B. subtilis* inhibiting the influenza colonies may be an example of a strong alkali former preventing the development of the bacilli as already discussed. On 1% fermentable sugar mediums pneumococci and streptococci still exert their favorable influence.

In connection with the experiments with laked corpuscles some of the blood laked by the addition of a large amount of water was passed twice through a Berkefeld filter. The filtrate, distinctly tinged red and proved to be sterile, was added to the plain medium plates which were inoculated with several strains of Pfeiffer bacilli. Not only did the bacilli grow well on this filtered hemoglobin medium, but the satellite arrangement appeared quite as well as on ordinary blood plates. It is clear from this that the growth factor is not held back or absorbed appreciably by the process of filtration.

The variety of blood used in plating appeared to make no appreciable difference. The phenomenon appears equally well with human, sheep, horse, rabbit, guinea-pig, dog and pigeon bloods. Pigeon blood yields the most profuse growth, and for that reason the satellitism is not so evident.

Washed red cells do as well as unwashed in the medium in revealing the phenomenon. This is an important fact because it excludes serum inhibition as a possible factor in the reaction. Pfeiffer bacilli are to some degree inhibited in their growth by blood serum. Several experiments were made by adding increasing amounts of serum to ordinary chocolate blood medium (heated to 90 C. for 5 minutes), on which an abundant growth appears. As the amount of serum increased, there was noticed some diminution in the amount of growth. In other words, chocolate agar to which unheated serum has been added is not as favorable a culture medium as chocolate agar without the serum. One might postulate that in the zone about the central colony the serum has been altered so that it has lost its inhibiting effect.

While this may or may not be true, it probably has little or nothing to do with this phenomenon since the experiment in which washed corpuscles were used in the medium conclusively demonstrates that the presence of serum inhibition plays no rôle in the reaction.

Experiments were designed to test the influence of other substances on Pfeiffer's bacillus. Masses of dead bacteria killed by heating or drying, or dying naturally in a test tube, were prepared, and a small amount placed on a blood plate seeded with Pfeiffer bacilli. Strains of staphylococci colon bacilli and streptococci, all of which when alive and growing on Pfeiffer plates revealed satellitism, showed no favoring influence when thus tested. This experiment was made many times, and no stimulating effect was ever noted by these dead bacteria. This merely indicates that from the dead bacteria the favorable substance or influence is not disseminated or diffused to any appreciable extent. When dead bacteria or filtrates of cultures are intimately mixed with blood medium, Pfeiffer bacilli will grow on this far more profusely than on medium without the bacteria. From the living and growing bacteria, however, the favorable substance is diffused into the surrounding medium for some distance.

Next sterile animal tissue was tried. Small pieces of guinea-pig and rabbit liver, kidney, myocardium, voluntary muscle, spleen and brain were freshly prepared and placed on a blood plate seeded with Pfeiffer bacilli. About such tissue definite satellitism was seen after 24-hour incubation, all tissues behaving essentially alike in this respect. Curiously enough, a drop of blood placed on a hemoglobin plate does not reveal this reaction, or at least only to a slight degree. For this reason, one can exclude the blood or hemoglobin that may be present in these tissues as the determining factor. When one heats these animal tissues in the autoclave or to boiling for some time, they will no longer show satellitism with Pfeiffer bacilli. So, too, small pieces of fresh carrot and potato obtained under sterile precautions will also reveal the phenomenon on blood plates, but when autoclaved for a short time will no longer do so. One may therefore state that the factor responsible for this satellite phenomenon is destroyed by heat. In all the experiments, of course, proper precautions were taken to avoid any bacterial contamination of the animal or plant tissue. They must be absolutely sterile.

I have tested many chemicals, including iron compounds, both organic and inorganic, oleic acid, etc., without finding one that will consistently do what the living colony or tissue will do. It should be

said that no chemical will act as a substitute for blood or hemoglobin in growing Pfeiffer bacilli.<sup>4</sup> Ghon and Preyss<sup>5</sup> showed that on hematin agar Pfeiffer bacilli will grow when associated with another organism but not alone. However, when placed on a blood plate, hematin will not show satellitism. Oleic acid has been added to mediums to advantage in growing Pfeiffer bacilli, especially in the last few years. When small droplets or pieces are placed on a blood plate which has been inoculated with Pfeiffer bacilli, a wide clear zone of hemolysis soon appears around it. Usually the bacilli are not stimulated to grow in this zone, but I have noted at times that near the outer margin of the hemolytic zone large Pfeiffer colonies may form quite comparable to those about a bacterial colony. Whether or not this phenomenon is comparable to that which appears about bacterial growth I am not able to say. Presumably, it changes the hemoglobin into a derivative (hematin), thereby permitting another substance in the blood, serum or in other bacteria to act with it and enhance the growth of the Pfeiffer bacilli. This substance I will discuss further.

The question arises as to the relation of this favoring influence of a foreign organism and the favoring influence of heated hemoglobin on the growth of Pfeiffer bacilli. It has long been known that heating blood medium—that is, chocolate medium—improved it for growing this bacillus. I have studied this phenomenon,<sup>6</sup> and it appears that in order to obtain the most favorable medium the heating of blood must be done between quite definite limits—55 and 120 C., and for definite periods of time. Blood heated to 55 C. indefinitely will not yield a maximum growth. Heating to 60 C. for 2 to 4 hours is necessary, but if continued for 3 or 4 days, the medium is rendered useless. Boiling the blood medium for a moment will yield a favorable medium, but boiling for 1 to 2 hours will destroy its value for Pfeiffer bacillus cultures. Autoclaving for 30 minutes will also destroy its value. These results may be plotted in the form of a curve.

The fact that superheating or autoclaving will render blood mediums valueless for the culture of Pfeiffer bacillus permits one to analyze the satellite phenomenon further. On an autoclaved blood plate Pfeiffer bacilli will not grow. Mixed with another organism it grows in the zone about the foreign colony very well, thus showing satellitism on the autoclaved medium even better than on unheated blood medium. Moreover, by adding a bacterial filtrate or a carrot or potato filtrate

<sup>4</sup> Jour. Infect. Dis., 1907, 4, p. 73.

<sup>5</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1897, 25, p. 453.

<sup>6</sup> Jour. Infect. Dis., 1921, p. 169.



(Berkefeld) or blood serum to such filtrated medium, Pfeiffer bacilli will grow profusely, an excellent medium thereby being furnished for the cultivation of this organism.

The appearance and characteristics of the zone on this medium are identical with those described on unheated plates. Owing to the clots of blood formed in the heated medium, in order to observe the satellitism, it is necessary to remove them before plating. This can readily be done by passing the medium through filter paper when hot. Using this filtered blood medium and inoculating with Pfeiffer's bacilli, the phenomenon is satisfactorily observed about a foreign colony or other material used for this purpose.

From this last observation we may conclude that we are dealing with two substances, one of which is heat stabile and is present in the heated blood, the other a substance less heat resistant and found in extracts and filtrates of animal and plant tissues of bacterial cultures. These two substances when operating together in plain medium will allow a profuse growth; either one alone will not permit growth in plain medium. This conclusion has been arrived at and developed in a previous article<sup>6</sup> through a study of the growth of this organism on autoclaved blood medium in tubes to which various activating substances were added. The results there presented agree entirely with those reported here and enable us to explain satisfactorily the satellite phenomenon. On the autoclaved blood medium plates seeded with Pfeiffer's bacilli growth will appear only around a foreign colony where the heat labile body necessary to complement the heat stabile body of the heated blood is generated. This substance from the foreign colony or from a piece of carrot or animal tissue is a diffusible substance, for it exerts its influence to a distance the width of the satellite zone. On unheated blood plates the process is more complex because, in addition to the hemoglobin and its derivatives, there are other compounds in the serum and even in the corpuscles. It appears, however, that the foreign colony not only furnishes the heat labile substance necessary, but also breaks up the hemoglobin into its derivatives (hematin and hemin) with which the former acts. It is difficult to be certain of this last point because hemoglobin is an unstable substance, and it is difficult to know when it is free from its derivatives. Probably in blood plates there is always some hematin, and it may very well be that pure hemoglobin or oxyhemoglobin will alone (in plain mediums) not support the growth of Pfeiffer bacilli. This would seem to be indicated by the fact that in crystallized hemoglobin medium

or in fresh blood medium the growth of Pfeiffer bacilli is scant and even at times negative. In all probability, then, a foreign bacterium or animal or plant tissue on unheated blood plates breaks down the hemoglobin into its derivative hematin, at least to some extent, and also furnishes the heat labile substance referred to. Thus, in a uniform zone about bacteria, tissue, etc., exist the two substances the cooperation of which is necessary for the profuse growth of Pfeiffer bacilli.

A final point deserving a word of comment is the possible influence of another organism on the growth and possibly on the virulence of Pfeiffer's bacillus in the animal body. The statement has been made that in mixed culture Pfeiffer's bacillus is more dangerous and more pathogenic for animals and possibly for man. Some years ago I made experiments on animals which seemed to indicate that this is true, and it may be true to some degree. However, we now know that various living tissues of the animal will do exactly what an associated organism will do in the way of stimulating the growth of Pfeiffer's bacillus, so that apparently this favoring influence is being exerted by various tissues of the body on this bacillus during an infection, and the additional influence of an associated organism, as for example streptococci or staphylococci in the respiratory tract, would presumably make little difference in the final result.

#### SUMMARY

The phenomenon of satellitism as observed in connection with Pfeiffer's bacillus is described in detail.

It is observed in association with bacteria, yeasts and fungi of various kinds and their filtrates; also with plant and animal tissues and their extracts and filtrates.

Heating in the autoclave (120 C. for 30 minutes) will destroy the activities of these substances. Heating for longer periods at lower temperatures will do likewise.

On clarified autoclaved blood medium Pfeiffer's bacillus will not grow. On plain medium, to which organisms or tissues or their filtrates (hemoglobin free) are added, it will not grow. When these substances are mixed good growth results.

The explanation of satellitism therefore would seem to be: Diffusible products or extracts of bacteria, fungi, tissues, etc., stimulate the growth of Pfeiffer's bacillus in conjunction with hematin or with hemoglobin. Thus, profuse growth of Pfeiffer's bacilli occurs immediately around colonies of organisms or pieces of plant or animal tissue.

# THE ACCESSORY FACTORS IN BACTERIAL GROWTH

## V. THE VALUE OF THE SATELLITE (OR SYMBIOSIS) PHENOMENON FOR THE CLASSIFICATION OF HEMOPHILIC BACTERIA

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I have pointed out<sup>1</sup> that the satellitism of Pfeiffer's bacillus may be observed with many diverse micro-organisms. This suggests the possible value of this phenomenon for purposes of classification and identification of the hemophilic bacteria. Indeed, in my own work I have used this test for some time and I long since concluded that it was one of the most reliable and uniform criteria we have for this group.

The value depends on two facts: First, apparently all strains of the Pfeiffer type of hemophilic bacilli will reveal the satellite phenomenon when grown with another organism on blood plates. I have tested many hundreds of strains and have yet to find an exception. Second, the important point, which I have only recently observed, that Pfeiffer bacillus does not favorably influence itself. That is, if one prepares a blood plate seeded diffusely with Pfeiffer bacilli and then inoculates here and there with the same organism, after incubation one will observe no favorable influence on the colonies of the organisms last inoculated.

It seemed to me that in view of certain slight differences between strains of this group, cultural and otherwise, that possibly here was a method of further differentiation. Accordingly, using as central colonies various strains of Pfeiffer bacilli, blood plates were seeded with homologous and heterologous strains. Strains thus tested included 3 from the lungs of patients with epidemic influenza pneumonia, 1 from influenzal meningitis, 3 from excised adenoids and 1 from an infected frontal sinus; also 3 indol and 3 nonindol forming strains obtained from Dr. E. O. Jordan. In no instance, when such "cross" satellite tests were made, did there appear any favoring influence of one strain

Received for publication April 18, 1921.

<sup>1</sup> Jour. Infect. Dis., 1921, 29, p. 178.

on another, though all were influenced favorably by staphylococci and other bacteria. Various strains were mixed on the same medium and cultivated together to find whether the growth was enhanced to any degree. The results were negative. It would appear, then, that the differences that have been noted by agglutination, cultural reactions and virulence tests between strains of Pfeiffer bacilli cannot be detected by this test.

However, for differentiating from closely related groups, the test becomes of real value. This applies especially to the organisms that grow well on blood and now commonly classed as hemophilous. The Committee on Classification of the Society of American Bacteriologists has recently placed provisionally in the genus *Hemophilus*, the Pfeiffer bacillus, *B. pertussis* (Bordet), the Morax-Axenfeld bacillus, *B. ducreyi* and the Koch-Weeks bacillus. Strains of all these organisms were subjected by me to this test of satellitism. They were used as central colonies on plates seeded with Pfeiffer's bacilli and about all, a favorable influence on the Pfeiffer bacilli was noted. Three strains of *B. pertussis*, one of Morax-Axenfeld and one of *B. ducreyi* were used. On the other hand, none of the bacilli just mentioned is favorably influenced by other bacteria. It appears then that here is a definite and sharp method of differentiating the Pfeiffer group of bacilli from other hemophiles.

It should be stated that apparently none of the latter organisms is strictly speaking hemophilic as is the Pfeiffer bacillus. While they grow better on blood or hemoglobin medium, they can all be cultivated on special mediums without blood. Another difference between the Pfeiffer bacillus and the other bacteria mentioned is that the former grow and indeed grow better on medium containing small even minute quantities of hemoglobin, whereas the latter require large amounts of blood or tissue fluids.

Several strains of bacilli from the conjunctiva, which, I think, commonly would be called Koch-Weeks bacilli, were tested and none revealed satellitism with Pfeiffer bacilli. On the other hand, they were favorably influenced by other organisms (staphylococcus and streptococcus) exactly as are typical Pfeiffer's bacilli. I am therefore inclined to the view that they all are strains belonging to the Pfeiffer group. This test would not exclude, of course, the possibility of their being differentiated from other strains by finer methods.

## SUMMARY

The satellite phenomenon is of value in identifying and in classifying members of the hemophilic group. Its value depends on the fact that Pfeiffer's bacillus is not favorably influenced in its growth by homologous or heterologous strains, while apparently all other strains of hemophilic organism reveal this phenomenon.

# CHANGES IN THE HUMAN CENTRAL NERVOUS SYSTEM IN BOTULISM

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Death from food poisoning has long been known. About one hundred years ago it was ascribed to metallic poisons from kitchen utensils. In 1820, Kerner,<sup>1</sup> in describing a severe outbreak of food poisoning from eating smoked meat and sausage, was the first to apply the name "ptomaines" to the putrefactive poisons formed in organic substances; forty years later *Trichinella spiralis* was found contaminating food and shortly afterward the bacterial origin of food poisoning was announced by Gaertner, Gaffky and Paak and Achard and Bensaude.<sup>2</sup>

Of all the organisms found in food *B. botulinus*, through its toxin, is the most harmful for man and animal. It was first described by Van Ermengem,<sup>3</sup> who discovered it in a ham, the eating of which led to fifty deaths.

Since that time many contributions have been added to our knowledge of botulism, and it has been established that the bacillus botulinus is found not only in sausage, as its name implies, but also in other foods. Two deaths from drinking clam-juice have been reported;<sup>4</sup> three from eating salted herring;<sup>5</sup> three from cheese<sup>6</sup> and a great number from spoiled meat and vegetables.<sup>7</sup>

During the recent outbreak of food poisoning from canned olives in various parts of this country, especially in California, the clinical symptoms and morphology of the organism have again been described, and a few reports on the pathologic changes in the human body have been added.

The following report deals with the study of a brain-stem examined in serial sections from a case of botulism in a girl, 17 years old.

During Nov., 1919, 5 persons became sick in Sioux Rapids, Iowa, from eating smoked ham and salted pork, kept in a barrel. For the details and careful observation of the illness of all these I am greatly indebted to Dr. E. E. Munger of Spencer, Iowa. The meat was eaten in a farm-house close to the village and at least 2 of the 5 persons, one of whom died, were taken ill abruptly after leaving the farm-house where they had been stopping for a short time. Except for a nurse, these were all of one family: two—mother and daughter—died. Regarding the nature of the third death, that of the nurse,

Received for publication April 23, 1921.

<sup>1</sup> Monograph, 1820.

<sup>2</sup> Cited by Hühner, *Ergebn. d. inn. Med. u. Kinderh.*, 1912, 9, pp. 30-102.

<sup>3</sup> *Ztschr. f. Hyg. u. Infektionskrankh.*, 1897, 26, p. 1; *Arch. de pharmacodyn.*, 1897, 3, p. 204; *Handbuch der pathog. Micro-organ. Kolle-Wassermann*, 1912, 4, p. 909.

<sup>4</sup> Bine, R.: *Boston Med. & Surg. Jour.*, 1917, p. 559.

<sup>5</sup> Bitter: *Deutsch. med. Wehnschr.*, 1919, 45, p. 1300.

<sup>6</sup> Nevin: *Jour. Infect. Dis.*, 1921, 28, p. 226.

<sup>7</sup> Schede: *Med. Klin.*, 1916, 12, p. 1309. Dickson: *Jour. Am. Vet. Med. Assn.*, 1917, 69, p. 966; *Calif. State Jour. Med.*, 1916, 14, p. 143; *Proc. Soc. Exper. Biol. and Med.*, 1916, 14, p. 47. McCaskey: *Am. Jour. Med. Sc.*, 1919, 57, p. 158. Emerson and Collins: *Jour. Lab. & Clin. Med.*, 1920, 5, p. 559.

brought from Sioux Falls, some doubt exists because she left this place on Nov. 8, became suddenly ill Nov. 15 while caring for another patient in South Dakota, and died 22 hours after the onset of the illness. One of the relatives, a sister-in-law, was only mildly ill for a few days. Nov. 5, she noticed that some of the pork given her was spoiled and threw it away. She had received the pork at the farm-house from the same barrel. Several others were ill at this time but with symptoms not definitely of botulism.

The illness of a fifth patient, a man, S. A., began Nov. 5 with a dry throat and mouth, dysphagia and restlessness; delirium became marked, and he talked for 5 hours at one time; he complained of headache, pain in the legs and back of the neck; there was a sighing respiration with distinct air hunger; blood pressure on Nov. 12 was 125 systolic and 85 diastolic. He improved gradually, and Dec. 25 had practically recovered.

The initial symptoms of the illness of the daughter, 17 years old, were great dryness of the mouth and throat; later she perspired freely, complained of sore throat, pain in the back of the neck and ringing in the ears. She became very restless and moaned and groaned constantly. There was edema of both sides of the face and neck. A bright red spot developed on the left shoulder which was very tender. Nov. 13, she menstruated; at this time the blood pressure was 170 systolic and 120 diastolic; she also complained of pain in the eyes. Nov. 22 a soft systolic murmur developed at the apex. She gradually became more restless, talkative, at times delirious, and was not influenced by bromides or codein. The temperature during the course of the disease ran from 98 to 104.2 F.; the pulse rate from 60 to 160, and respiration from 18 to 46. The striking symptoms were rigidity of the neck, severe pain in the head, neck, shoulders and abdomen, irrationality, unconsciousness, restlessness and frequent involuntary micturition and defecation. In addition, there were muscular twitchings, knotting of the muscles and strabismus of the right eye. The blood pressure was 150 systolic, 130 diastolic, hemoglobin 80 to 90%. The pulse was dicrotic, frequently weak and irregular and at times there were Cheyne-Stokes respirations. Pressure on the back of the neck caused dizziness. Dec. 16 her pulse was 120, temperature 99.6 per rectum; she was very sore all over; vomiting frequent; there was slight ankle-clonus of both legs; the knee-jerks were absent or very faint. At one time there was paralysis of the left external rectus and slight involvement of the muscles of the face and right side. Subsequently (Dec. 16) movements of the left eye were normal and the facial muscles nearly so, but there was complete paralysis of the right external rectus. The blood pressure was 140 systolic and 120 diastolic; the mind was fairly clear, with occasional delirium and involuntary urination; marked leukocytosis, with a high eosinophilia, was present.

Dec. 21, at 1:30 p. m., the patient became rigid with slight convulsive twitchings of the muscles after a paroxysm of pain in the head, neck, right arm and shoulders. The head was bowed back, jerking; then she would bury it in the pillow. She became unconscious following a chill. At this time the temperature was 104, the pulse rate 160. Dec. 22 another paroxysm of pain came on, but this time without convulsive twitching of the muscles. At 4:45 a. m. the following day another paroxysm of pain came on, and this time the patient rolled from side to side in bed. At 6:30 a. m. on the same day these paroxysms of pain became more frequent and of longer duration, and at this time the patient cried out with extreme pain in the head, twitching of the muscles, followed by a short chill; just before the chill she became unconscious. Such paroxysms became more frequent, and Dec. 26 they were so severe that the muscles all over the body twitched, throwing her legs from

one side of the bed to another. Dec. 27, at 8:45 a. m., she turned over on the left side with the head and feet thrown back for one-half minute; this was followed by 8 minutes of muscular twitchings and trembling of the lower extremities; death occurred on the same day.

NECROPSY (Dr. E. R. LeCount): Outside of the central nervous system no appreciable change was noticed in any organ that might be ascribed to botulismus, and therefore only the anatomic diagnosis is given here: Fibrino-

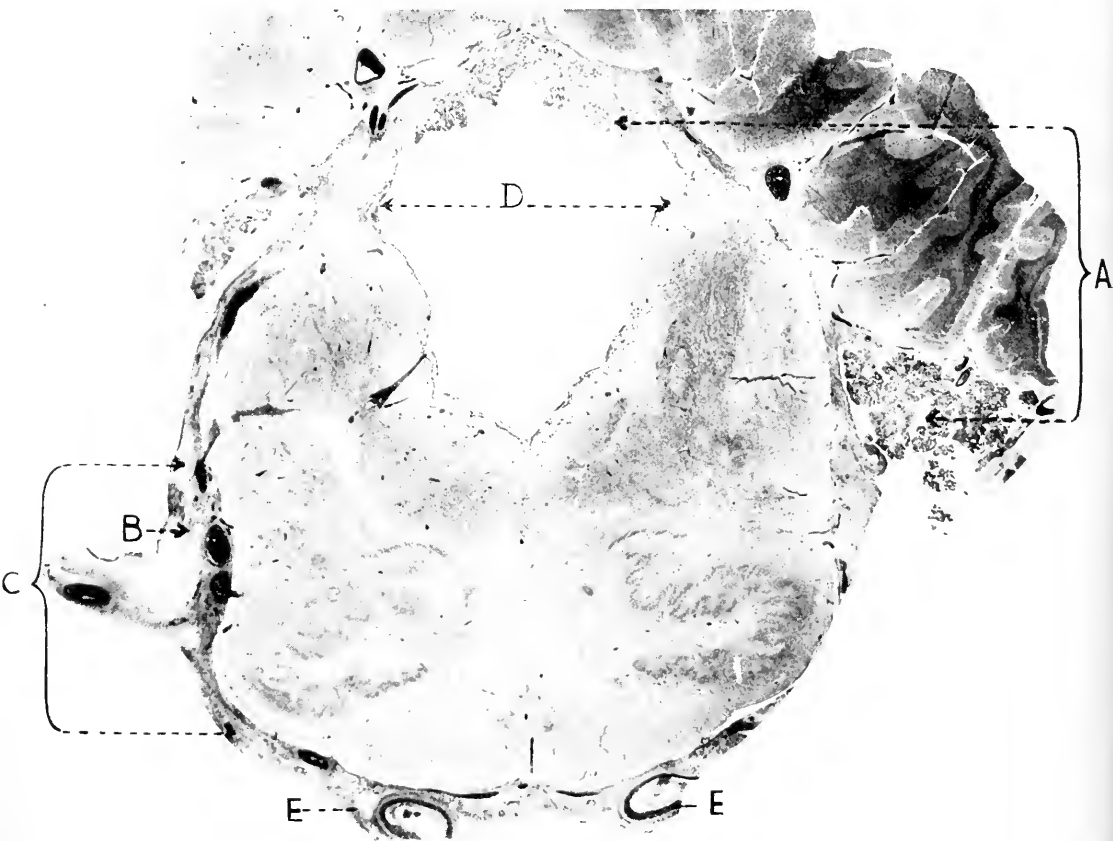


Fig. 1.—Camera lucida; photomicrograph; X 5. Section through middle of olives. A, plexus chorioideus densely infiltrated with lymphocytes; B, NN. facialis and acoustic free of infiltration; C, densely infiltrated and thickened pia-arachnoid; D, lymphocytic infiltration of subependymal layer; E, endarteritis in vertebral arteries (recently organized thrombus).

purulent basilar leptomenigitis; marked edema and anemia of the brain; anemia of the leptomeninges; foramen-magnum-pressure-furrow of the cerebellum; moderate internal hydrocephalus; marked general anemia; cloudy swelling of the liver and kidneys; fatty changes of the liver; hypostatic hyperemia of the stomach, urinary bladder and duodenal mucosa; moderately



distended bladder; slight hyperplasia of the biliary lymph glands and of the thyroid gland; left fibrous pleuritis; fibrous adhesion between the mesosigmoid and left ovary; localized hyperemia of the back (beginning pressure necrosis); moderate general obesity; accessory furrow of the under surface of the right lobe of the liver; fat replacement of the axillary lymph glands.

The calvarium is easily removed. In the longitudinal sinus there is a little fluid blood. There is practically no space between the two layers of the arachnoid. The outside of the cerebellum is rather dry. The cerebral veins are flat and mostly empty, both in front and behind. There is a slight but definite gray, purulent exudate covering the basilar and vertebral arteries. The ventricles of the brain are full of fluid. The region behind the chiasma

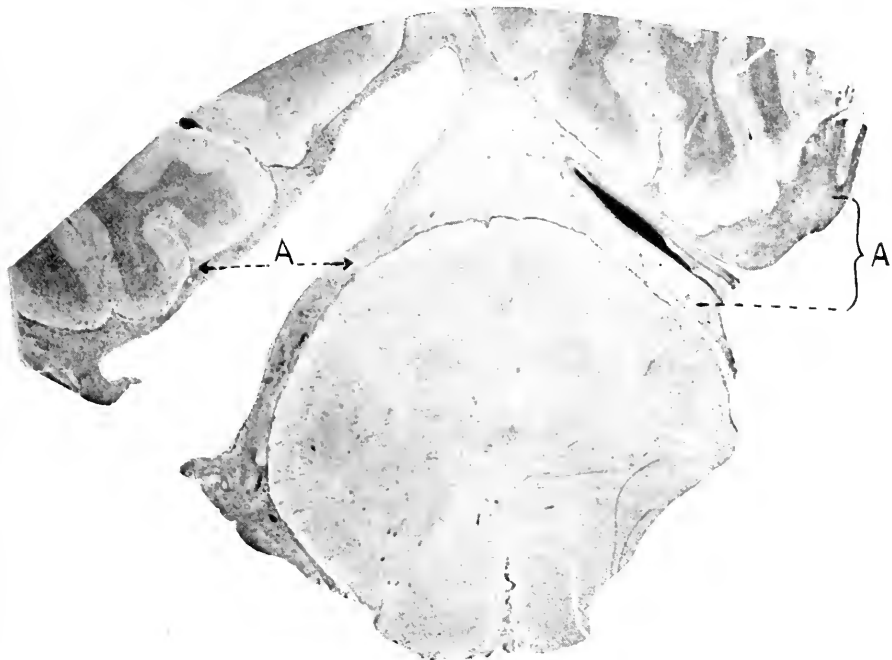


Fig. 2.—Camera lucida; photomicrograph; X 5. Section of medulla at decussation of pyramids. A, densely infiltrated and thickened pia-arachnoid.

bulges as though the third ventricle was full of fluid, and the tissue limiting this fluid on the bottom of the brain is translucent. The under surface of the temporal and occipital lobes is flat and semifluctuant from fluid in the ventricles. There is an exudate filling the tissue behind the medulla and in the region of the cisterna. In the substance of the cerebellum there is no change except that the vessels are rather bloodless. The lining of the left lateral ventricle is smooth and wet, and the ventricle is a little enlarged, the brain tissue about it soft. In the posterior horn of this ventricle the lining is greenish from exudate. The cerebrospinal fluid is distinctly turbid. The condition in the left hemisphere and ventricle is similar to that of the right. The substance of the cerebrum is watery and soft; the blood vessels empty. There is

no focal lesion in the brain. In the sinuses of the back of the dura there is only fluid blood. There is a brown fluid in the middle ear; the membrane here is intact. There is gray mucus in the sphenoid sinus with a thin, pink fluid, and a similar fluid in the ethmoid and frontal sinuses. There is no gross change in the hypophysis except that the substance is wet.

The whole brain-stem with the medulla, the pons, a portion of the cerebellum and spinal cord, was removed, sectioned serially and stained by the Weigert-Pal method, eosin-hematoxylin, phosphotungstic-acid-hematoxylin, and Nissl stains, as well as by stains to demonstrate bacteria. Sections were also taken from the central nuclei.

In sections stained by the Weigert-Pal method there is no change in the myelin sheaths. The fibers stand out sharply against the gray matter throughout. The eosin-hematoxylin stain reveals the changes best. They consist of a dense lymphocytic infiltration of the bulbospinal pia-arachnoid, the subepen-



Fig. 3.—Photomicrograph; X 30. A, endarteritis of basilar artery; B, densely infiltrated and vascular pia-arachnoid.

dymal layer, the tela choroidea, tegmentum and choroid plexus (Fig. 1). At the lowest level of the olives and about the spinal cord the leptomeninges are four times the normal thickness (Fig. 2). The infiltration in the pia-arachnoid, profuse throughout, is especially dense about its blood vessels, both arteries and veins. About the capillaries and arterioles of the floor of the fourth ventricle and aqueduct of Sylvius, they form dark specks, easily seen with a hand-lens, 3 diam. (Fig. 1). The lumen of the basilar and vertebral arteries is partly obliterated by a circular bundle of long, spindle-shaped cells with elongated, dark-staining nuclei, recently organized granulation tissue forming a circular bundle of from 20 to 30 cell-layers, closely attached to the internal elastic membrane (Fig. 3). This endarterial thickening is readily followed through the whole length of the vertebral arteries and the basilar artery, obliterating from one third to one half of their lumen, and a similar thicken-

ing of the intima can be followed in at least three of the medium sized branches. Most of the smaller arteries and arterioles are filled with lymphocytes and some desquamated endothelial cells, but in a few arterioles the endothelial leukocytes predominate. The walls of all the vessels are infiltrated with lymphocytes which also fill the perivascular space; veins and arteries are equally involved. In the veins and arterioles all the coats are uniformly affected, whereas in the larger arteries the media is comparatively free of inflammatory changes and lymphocytic infiltration. The acoustic area is densely infiltrated, the lymphocytes in that region pervading the central gray matter. All the minute arterioles along the raphé are almost obliterated by lymphocytes, but other vessels in the substance of the medulla and brain-stem have hardly a sprinkling of lymphocytes in or about them, and the majority are entirely free



Fig. 4.—Photomicrograph; X 30. A, tegmentum markedly infiltrated with lymphocytes.

from such alterations. The ventral pia-arachnoid, especially in the neighborhood of the basilar and vertebral arteries, is extremely vascular (Fig. 3). A mass of capillaries with projecting protoplasmic buds are seen, irregular in size and shape, some of them communicating with each other and giving the appearance of granulation tissue. The infiltration in this region is profuse, the lymphocytes being closely packed between the capillaries. In sections taken, at the postmortem examination, from the lining of the ventricle where there was so much exudate, in places greenish, there is a great deal of edema and the greater portion, about 2 cm. by 4 mm., is fenestrated from necrosis and the cells are scanty. In places there is only a fibrillar network with scattered gliacells and nuclear material. This necrotic region involves both the white and gray matter (Fig. 5). All the vessels in the subependymal layer are densely

infiltrated with lymphocytes and to a lesser degree with plasma cells and endothelial leukocytes. This infiltration also fills the spaces about these vessels and has also invaded considerably the surrounding subependymal tissue (Fig. 5). The veins are filled with erythrocytes, while the arteries are empty as a rule. The lumen of most of the small arterioles is densely packed with lymphocytes. In the center of two veins are masses of fibrin. The larger ganglion cells in the non-necrosed region stain deeply; they have swollen and pale nuclei, bulging in many instances at the periphery of the cell.

The larger neurons of the medulla, pons and cerebellum are normal; the Purkinje cells with their Nissl granules are unaltered. The folds of the pia-arachnoid, which dip into the cerebellar fissures, are also densely infiltrated with lymphocytes and contain engorged veins. Careful examination of the

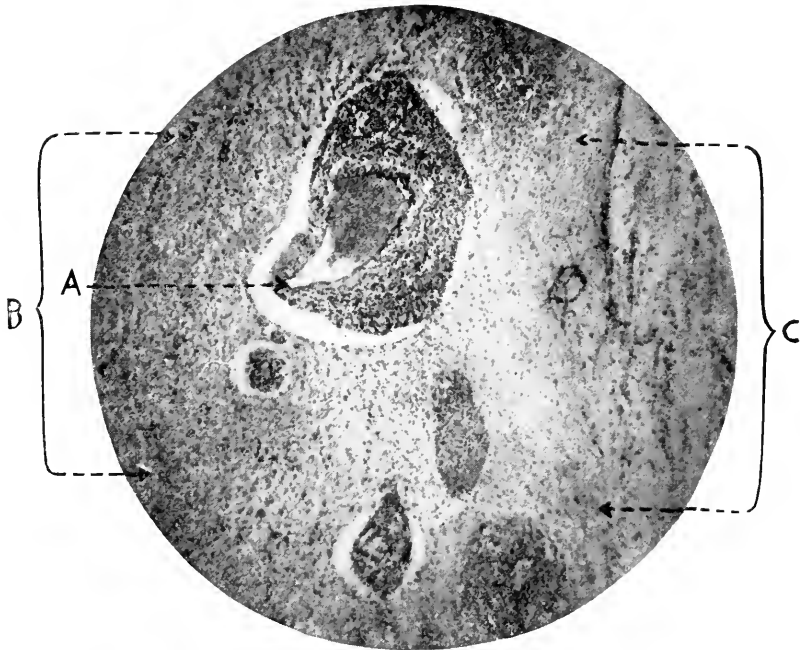


Fig. 5.—Photomicrograph; X 65. A, marked perivascular lymphocytic infiltration; B, dense lymphocytic infiltration of subependymal layer; C, region of extensive ischemic necrosis.

cells of origin of the cranial nerves does not reveal any characteristic change. The cell-bodies are uniformly swollen, with pale-staining, large nuclei and distinctly staining nucleoli. Only the uppermost cells, those nearest the ependyma, of the oculomotor and trochlear nuclei, in the immediate neighborhood of some densely infiltrated vessels, are irregular in shape, the Nissl granules faint or invisible, and of some of the ganglion-cells only a faint outline can be distinguished. Here the lymphocytic infiltration is not only about the vessels, but also diffused in the central gray substance and the tegmentum at the decussation of the trochlear nerve. In the section through the distal end of the nucleus ambiguus, the edema in the medulla is more pronounced, the

pia thicker and more densely infiltrated with lymphocytes. The aqueduct of Sylvius is distended, and its ependymal lining as well as that of the 4th ventricle is somewhat desquamated, especially from the floor. The pia arachnoid of the cervical portion of the spinal cord is thick and densely infiltrated with lymphocytes and also a considerable number of plasma cells. The spinal cord and its vessels are free from lymphocytes, except the end arterioles along the ventral and dorsal fissures, which are also densely infiltrated and their lumen almost entirely obliterated by lymphocytes. The cranial and spinal nerves are not involved in the infiltration; the nerve-trunks can be clearly seen traversing the meninges with hardly any infiltration about them.

A careful examination of sections of the heart, lungs, liver, spleen, pancreas, kidneys and lymph glands was also made; they were quite free of disease; none of the arterioles show any thickening of the intima and in many of these sections there are small arteries comparable in size with those in the brain-stem where the recently organized thrombi obliterate the channels in part.

Histologic Anatomic Diagnosis: Subacute bulbospinal meningitis, choroiditis and ependymitis; endarteritis (recently organized thrombi) of the basilar and vertebral arteries and their branches; edema of the brain and the leptomeninges; internal hydrocephalus; slight ischemic necrosis and pressure atrophy in the motor cells of origin of the cranial nerves; marked ischemic necrosis about the third ventricle; thrombosis of ependymal veins.

Bacteriologic Examination (Dr. S. J. House): At the postmortem examination material from the exudate at the base of the brain and in the ventricles was put into 4 blood-serum slants; cover-glass preparations from these revealed no bacteriae; on blood-agar plates incubation with material of these 4 tubes yielded no growth; moreover, in these tubes after 7 days at 37 C. no growth developed. Material of these 4 tubes was also transferred to medium maintained for 5 days at 37 C. and 2 days at room temperature under anaerobic condition; no growth developed. Efforts to obtain growth from the spleen under both aerobic and anaerobic conditions were negative.

Although several weeks had elapsed since the outbreak and considerable of the ham and salted pork had been thrown away or otherwise disposed of, cultures were made from a number of places of what remained with negative results.

In microscopic preparations from a number of skeletal muscles and brain tissue taken at the necropsy, no bacteria were found.

Animals were inoculated with pieces of the pork taken from several places, but no deleterious effects were observed. Not only was a prolonged search made for trichinella in the pork and ham which remained in the barrel, but also in such places as the diaphragm, laryngeal muscles and other muscles of the body of the patient; none was found.

#### DISCUSSION

Taking into consideration the illness in several persons after partaking of the same food, the similarity of the clinical course and symptoms and their correspondence with the changes in the central nervous system, the exclusion of trichinosis, the absence of the symptoms of ptomaine poisoning, especially of bowel disturbances, also the fact that the alterations in the brain-stem do not correspond to syphilis, epidemic encephalitis or to other known forms of disease, as well as

that they do resemble what is at present known in regard to the changes produced by botulismus, the conclusion seems justifiable that the illness in at least four of the persons mentioned was due to botulismus.

It is well established that the contamination of food with *B. botulinus* may be rather widely disseminated,<sup>8</sup> and unless such scattered foci in the food are examined or used for animal experiments, the nature of the contamination may not be ascertained.

There are reports<sup>9</sup> of the recovery of *B. botulinus* from the human spleen; on the other hand, failure to obtain the organism from the organs of animals experimentally inoculated are also recorded.<sup>10</sup>

The pathologic changes in the central nervous system of animals have been described in many instances. Minute disseminated hemorrhages in the brain-stem and medulla, especially in the floor of the fourth ventricle, with degenerative changes in the ganglion cells of origin of the motor cranial nerves, have been reported by Van Ermengem;<sup>3</sup> Roemer and Stein<sup>11</sup> and Marinesco.<sup>12</sup> Kempner and Pollack<sup>13</sup> found numerous hemorrhages in the anterior horn of the spinal cord. Intra- and peri-vascular lymphocytic infiltration has been noted by Ossipoff.<sup>14</sup> In most of these reports emphasis is laid on the degenerative changes in the ganglion cells of origin of the cranial nerves or in the anterior horn of the spinal cord. Graham and Bruckner<sup>10</sup> found a meningitis grossly during an epidemic in horses, *B. botulinus* being recovered from the ensilage which had served as food.

Reports on the changes in man vary to a certain extent. Brownlie<sup>15</sup> found a basal meningitis with hyperemia of the brain and spinal cord as the only change in one case (microscopic examination not reported). Concerning the material sent by Fischer<sup>16</sup> to the Pathologic Institute at Giessen (7 cases, 6 deaths), the report stated that no characteristic changes were found. In the examination made by Kellert and Nevin,<sup>6</sup> the pia at the base of the brain was thick and opaque; a few lympho-

<sup>8</sup> Schlossberger: *Arch. Pathol. Anat.*, 1854, 11, p. 569. Kaatzer: *Deutsch. med. Wehnschr.*, 1881, 7, p. 73. Seyler, Hoppe (cited by Eulenburg): *Realencyclop. d. gesamm. Heilk.*, 1890, 21, p. 369.

<sup>9</sup> Van Ermengem, Schuhmacher, Burger, Landmann (cited by Schede, footnote 7).

<sup>10</sup> Graham and Bruckner: *Jour. Bacteriol.*, 1919, 4, p. 1.

<sup>11</sup> *Arch. f. Ophth.*, 1897, 5, p. 43.

<sup>12</sup> *Presse Med.*, 1897, 5, p. 41.

<sup>13</sup> *Deutsch. med. Wehnschr.*, 1897, 23, p. 505.

<sup>14</sup> *Ann de l'Inst. Pasteur*, 1904, 58, p. 297.

<sup>15</sup> *Brit. Med. Jour.*, 1918, 1, p. 617.

<sup>16</sup> *Ztschr. f. klin. Med.*, 1916, 59, p. 58.

cytes were present in the anterior horn of the spinal cord and shrunken ganglion cells with swollen nucleus. Burger,<sup>17</sup> who made several post-mortem examinations, mentions changes in one brain; these consisted in dust-like desintegration of the Nissl granules and excentric position of nuclei, in the ganglion cells of origin of the oculomotorius. Paulus<sup>18</sup> described the changes in one brain; he found numerous foci of hemorrhage in the medulla and basal nuclei, especially at the floor of the fourth ventricle; there was no round cell infiltration or any sign of inflammation. The only reports I have found dwelling at any length on the microscopic changes are by Ophüls<sup>19</sup> and Dickson,<sup>20</sup> who both found perivascular hemorrhages and thrombosis of the arteries and veins in the brain and leptomeninges. In their instances the illness had lasted from 3 to 13 days. The longer duration of the illness, 6 weeks in the case here reported, may account for the more marked cellular exudate in the leptomeninges, as well as for the partial obliteration of the arteries.

Dickson's extensive experiments on 30 guinea-pigs, 37 rabbits, 30 cats and 4 dogs further convinced him that the pathognomonic change in botulismus is thrombosis secondary to some alteration of the intima, in man as well as in animals.

#### CONCLUSIONS

*Bacillus botulinus* produces a poison which is highly toxic to man and animal.

The changes are confined to the vascular system. Thrombosis in arteries and veins is the initial change followed by ischemic necrosis and later by inflammation.

The poison has no direct action on the nerve cells; the retrogressive changes are secondary and due to the disturbed blood supply.

The ganglion cells of origin of the motor cranial nerves are always involved because their blood supply is derived from terminals of branches of the vertebral arteries which seem to be the seat of predilection of the thrombosis.

<sup>17</sup> Med. Klin., 1913, 9, p. 1846.

<sup>18</sup> Jour. f. Psychol. u. Neurol., 1915, 21, p. 201.

<sup>19</sup> Arch. of Int. Med., 1914, 14, p. 589.

<sup>20</sup> Rockefeller Inst. f. Med. Res., 1918; Monograph, p. 117.

## ANAPHYLAXIS REACTIONS WITH PURIFIED PROTEINS FROM MILK \*

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In the course of a study of the proteins of cow's milk by one of us (O),<sup>1</sup> four proteins were isolated in a high degree of purity, and their individuality established by means of the anaphylactic reaction. Evidence of the essentially chemical basis of immunologic specificity, as emphasized in our previous communications on the immunologic reactions of the vegetable proteins,<sup>2</sup> thus received further support. Our experiments were performed chiefly to determine the chemical individuality of the various protein preparations under investigation, and the completeness of their separation from one another. However, since these results had an important bearing on problems of immunologic specificity, numerous additional experiments have been made from this standpoint with these carefully isolated proteins.

As one of the most readily available protein-containing solutions, milk perhaps has been used in immunologic experiments with greater frequency than any other material except blood and egg white. The ease with which casein can be separated from milk in a relatively pure condition and its ready solubility in dilute alkali have made this protein particularly suitable for use when a pure isolated protein was desired. Hence many references are to be found in the literature dealing with the immunologic reactions of casein. It is evident, however, from the published accounts, that the casein used in much of this work was not so carefully purified as it should have been. Furthermore, in only a few instances in which the proteins of the whey<sup>3</sup> have been used, have these been separated from each other with the care necessary to secure sufficiently pure preparations. As we have found no complete review of the literature of the immunologic reactions of the several proteins of milk, we here present a brief synopsis in approximately chronologic order.

Received for publication April 27, 1921.

\* The expenses of this investigation were shared by the Carnegie Institution of Washington, D. C., and the Otho S. A. Sprague Memorial Institute of Chicago.

<sup>1</sup> Osborne and Wakeman: Jour. Biol. Chem., 1918, 33, pp. 7 and 243.

<sup>2</sup> Wells and Osborne: Jour. Infect. Dis., 1913, 12, p. 341; 1916, 19, p. 183.

<sup>3</sup> The term whey is here used to designate the solution of the milk solids from which casein has been removed by precipitation with acid.



## IMMUNOLOGIC EXPERIMENTS WITH MILK AND MILK PROTEINS

Although the earliest experimental work on immunologic reactions with protein solutions dates to 1897, apparently isolated casein was not used until 1901. Moro,<sup>4</sup> Hamburger,<sup>5</sup> and Levene<sup>6</sup> independently, and almost simultaneously, published observations on the precipitin reactions with casein. Moro reported that by immunizing with pure casein, from either human or cow's milk, he obtained a "specific lactoserum," but he did not describe its properties further. He also reported that precipitins for cow's milk gave reactions with goat's milk, but not with human milk, which was not in agreement with the earlier work on milk precipitins by Wassermann and Schütze.<sup>7</sup> The important observation was made that heating milk one-half hour at 100 degrees did not destroy its reactivity or its antigenic property.

Hamburger<sup>5</sup> found that "lactalbumin" (which included all the proteins in solution after removing the casein) could be distinguished from casein by the precipitin reaction. Antilactalbumin serum (or, more properly, antiwhey serum) gave reactions with whole milk and with lactalbumin, but not with casein, and anticasein serum reacted with whole milk and casein, but not with whey. He prepared his casein by precipitating with acetic acid and then washing the precipitate free from "albumin." He also used Schlossmann's method of filtering off the casein through a "Thonzellenfilter," and stated that the "albumin" in the filtrate behaved somewhat differently from that obtained by precipitating out the casein.

In connection with other work, Levene<sup>6</sup> merely reported that immune serum for milk gave precipitates with milk, casein, "milk albumin" and beef serum.

Fuld<sup>8</sup> stated that whey obtained by filtering milk through porcelain filters did not react with antimilk serum. Since this is not in harmony with observations by others, it probably means that his immunization was sufficient to produce precipitins only for casein, and not for the other less abundant milk proteins. His statement that heated milk or casein solution did not produce precipitins has been shown to be incorrect. His antiserum for cow's milk did not react with goat's milk or with isolated casein from human milk.

Müller,<sup>9</sup> in a study of the relation of specific antiserum precipitins to rennin, observed that antimilk serum did not precipitate casein except in the presence of calcium salts, although it united with the casein in their absence. He found no evidence that the precipitation of casein by antiserum was accompanied by the splitting off of proteins similar to those of whey. In another article<sup>10</sup> he stated that the products of digestion of casein with pepsin or trypsin did not produce precipitins either for themselves or for casein, although paracasein and iodocasein immunization gave precipitins that precipitated casein. Lactoserum did not precipitate paracasein.

Gengou<sup>11</sup> isolated casein, lactalbumin and lactoglobulin from cow's milk, finding that the casein and globulin gave complement fixation and precipitin reactions with antimilk serum, but the albumin did not react. The antiserum for cow's milk reacted with goat's and sheep's milk, and less strongly with human and horse's milk.

<sup>4</sup> Wien. klin. Wchnschr., 1901, 14, p. 1073.

<sup>5</sup> Ibid., 1901, 14, p. 1202.

<sup>6</sup> Med. News, 1901, 79, p. 981.

<sup>7</sup> Verein f. inn. Med., Verinsbeilage, July 26, 1900, p. 178; Schütze. Ztschr. Hyg. u. Infektionskr., 1901, 36, p. 5.

<sup>8</sup> Hofmeister's Beitr., 1902, 2, p. 425.

<sup>9</sup> Arch. f. Hyg., 1902, 44, p. 126.

<sup>10</sup> Centralbl. f. Bakteriöl., I. O., 1902, 32, p. 521.

<sup>11</sup> Ann. de Inst. Pasteur, 1902, 16, p. 734.

Schlossmann and Moro,<sup>12</sup> who discussed the methods used in the separation of milk proteins, recognized that the so-called milk "albumin" is a mixture of globulin and albumin, containing no phosphorus, which statement later studies have shown to be incorrect as far as globulin is concerned. They found also that the casein and albumin fractions each gave specific reactions for the species from which they were derived, and also that globulin from human serum reacted with antihuman-milk serum, but not with anticow's-milk serum. Antiserum for human milk gave precipitates with human serum.

Amberg,<sup>13</sup> who reprecipitated his preparations, found that the precipitin reaction with milk or casein did not depend on the presence of either inorganic or casein salts of calcium, thus contradicting Muller. He observed no differences between the reaction with anticasein and antimilk serums when used against casein, milk, or lactalbumin.

Fleischer<sup>14</sup> immunized animals with purified casein from animals of various species (rabbit, cat, dog, horse, goat, cow and human), and found that the antisera gave precipitin reactions with both the homologous and foreign caseins, although with quantitative differences in favor of the homologous caseins. Antiserum for cow's casein gave about two-thirds as much precipitate with goat's casein as with cow's casein, and a good precipitate was obtained with horse's casein, but only a trace with dog's, cat's and rabbit's casein, and none with human casein. Antiserum for human casein gave only relatively slight reactions with all the other caseins.

In their early studies of anaphylaxis, Rosenau and Anderson<sup>15</sup> made some experiments with milk, obtaining no reactions when cow's milk was injected into guinea-pigs that had been sensitized with either human or dog's milk, but positive results were given when the sensitization had been caused by sheep's milk. The first examination of isolated casein by means of the anaphylaxis reaction is reported by Besredka,<sup>16</sup> who stated that casein gave the same results as milk. The whey was found to sensitize to milk sometimes, but it did not always give reactions in animals sensitized to milk. Perhaps the difficulty here lies in the use of too small amounts of the whey ( $\frac{1}{20}$  to  $\frac{1}{4}$  c.c.), which has a very small protein content.

Wells<sup>17</sup> observed that purified casein was even more active in anaphylactic reactions than whole milk, for 0.1 to 0.25 gm. of purified cow's casein usually produced fatal reactions when injected into the peritoneum of sensitized guinea-pigs, while doses of 5 to 10 c.c. of whole milk containing 0.15 to 0.30 gm. of casein were seldom followed by fatal reactions. The activity of casein solutions was not materially impaired by heating at 100 degrees for 25 minutes. Animals sensitized to goat's milk casein reacted severely when given cow's casein, or conversely, which agrees with Bordet's results with whole milk, and Fleischer's precipitin reactions with goat's and cow's casein.

A related observation on the lack of species specificity with casein is furnished by Michaelis and Rona,<sup>18</sup> who injected cow's casein subcutaneously into dogs and guinea-pigs that had recently been actively secreting milk, and observed a notable swelling of the mammary glands, without marked constitutional symptoms. Some reaction was obtained in virgin animals. This they interpret as the production of a heightened secretory activity which induced

<sup>12</sup> München. med. Wchnschr., 1903, 50, p. 597.

<sup>13</sup> Jour. Med. Research, 1904, 12, p. 341.

<sup>14</sup> Roussky Wratsch., 1908, 7, p. 1638.

<sup>15</sup> Jour. Med. Research., 1907, 16, p. 391.

<sup>16</sup> Ann. Inst. Pasteur, 1909, 23, p. 166.

<sup>17</sup> Jour. Infect. Dis., 1908, 5, p. 480; 1911, 9, p. 147.

<sup>18</sup> Pflüger's Arch., 1909, 121, p. 163.

the mammary glands to excrete the injected casein, although in none of the animals did actual secretion of milk follow the injection. On the other hand, Felländer<sup>18a</sup> was unable to sensitize guinea-pigs to guinea-pig's milk or to extracts of their mammary glands.

Bachrach<sup>19</sup> failed to secure a good differentiation between casein, whey and beef serum by anaphylactic reactions. As he used the same sized doses for both sensitizing and intoxicating, his observations are not valuable.

The use of the complement fixation reaction with casein was introduced by Kollmeyer,<sup>20</sup> who obtained distinct differentiation of casein from whey by this method.

Graetz<sup>21</sup> studied particularly the relation of milk to colostrum, and the only feature of his work of interest in this connection is the evidence obtained that colostrum whey contains proteins which seem to be identical with those of the serum. Other workers have obtained evidence of similar significance, indicating the whey proteins to be closely related to the serum proteins. Thus, Bauer<sup>22</sup> concluded that casein can be differentiated by complement fixation from the protein of the whey; that casein of different species of animals gives common group reactions, as also do the whey proteins, but that only the casein can be differentiated from the serum proteins of the same species. Bauereisen<sup>23</sup> finds that the complement-fixation method, like the precipitin reaction, does not permit an absolute differentiation of milk proteins from other proteins of the same individual, but that both methods show casein to be much less closely related to the serum proteins than are serum proteins to whey proteins. He also recognized the probability that the failure to distinguish sharply between the isolated proteins depends on defects in the method of their preparation.

The quantitative differentiation of milk proteins demonstrable by complement fixation is great enough to be recognized by the less sensitive anaphylaxis reactions, as indicated by the results obtained by Kleinschmidt.<sup>24</sup> He separated casein, globulin and albumin from cow's milk, but in his description of the process used does not indicate that he reprecipitated his preparations. His "casein" was everything that came down with sufficient acetic acid; the "globulin" was the precipitate produced by saturating with magnesium sulfate after removing the casein with NaCl, and the "albumin" was the precipitate obtained by adding acetic acid to the filtrate from the globulin. He found that anaphylaxis reactions (intracardiac) gave distinct differentiation between these three proteins. Casein sensitized to itself as well as whole milk, but did not sensitize to beef serum, lactalbumin or lactoglobulin. Experiments in the reverse order were almost equally specific. There were some slight crossed reactions, especially between globulin and casein, and a distinct protection (anti-anaphylaxis) reaction in all cases, probably due to incomplete separation of the proteins used. Guinea-pigs sensitized with cow's casein did not react with human casein; no other foreign caseins were tried. Animals sensitized with either cow's lactalbumin or lactoglobulin reacted with bovine serum, and conversely; those sensitized with lactalbumin reacted with lactoglobulin, and conversely. These last results do not warrant the statement made

<sup>18a</sup> Ref. in *Ztschr. f. Immunität*, Ref., 1912, 6, p. 851.

<sup>19</sup> *Vierteljahr. gericht. Med.*, 1910, 40, p. 235.

<sup>20</sup> *Ztschr. f. Biol.*, 1910, 54, p. 64.

<sup>21</sup> *Ztschr. f. Immunität*, 1911, 9, p. 677.

<sup>22</sup> *Berl. klin. Wehnschr.*, 1910, 47, p. 830.

<sup>23</sup> *Ztschr. f. Immunität*, 1911, 10, p. 306.

<sup>24</sup> *Monatschr. f. Kinderheilk.*, 1911, 10, p. 402.

in the author's summary: "In anaphylaxis experiments the proteins of cow's milk, casein, albumin and globulin, can be differentiated from one another." There are quantitative differences in his reactions, to be sure, but those are not constant. It is quite evident that the author was working with imperfectly purified protein preparations. A similar difficulty is found in the work of Heuner<sup>25</sup> who used commercial (Merck) cow's milk albumin and casein, as well as a milk albumin and a globulin prepared by a chemist for him, the description not indicating that his preparations were thoroughly purified. Nevertheless, he also reached the conclusion that globulin and albumin of milk are distinct from casein, and that colostrum and mastitis milk are more nearly related to the blood serum antigens than is normal milk. The differentiation was less marked in anaphylaxis experiments than with complement fixation.

Bauer and St. Engel<sup>26</sup> separated casein, globulin and albumin in the same way as Kleinschmidt, but reprecipitated the globulin three times, although the albumin seems not to have been reprecipitated. Using the complement-fixation reaction, they found distinct quantitative differences between all three proteins. Thus, with an anticasein (bovine) serum, complement fixation was complete, or nearly so, in the following dilutions of 0.5% solutions: casein, 1:256; globulin, 1:32; albumin, 1:16; milk, 1:64. With antiglobulin serum the figures were: casein, 1:8; albumin, 1:32; globulin, 1:16,384. With anti-albumin serum the figures were: casein, 1:1; globulin, 1:8; albumin, 1:64. Similar results were obtained with the globulin or casein of human milk. The proteins of one species were not tested against the antisera for the other species. Cow's milk globulin could not be differentiated from beef serum globulin, hence these authors believed the globulin and albumin of milk or colostrum or serum to be identical.

Klein<sup>27</sup> made a study of the nature of casein antiserum by the precipitin test, finding evidence that there are in such an antiserum two precipitins. One acts only in the presence of  $\text{CaCl}_2$ , while the other acts best in the absence of calcium. Hence the presence of two different antigen groups is postulated in the casein molecule. So far as we can learn, this important work has not been reinvestigated. The suggestion of the existence of two antigenic groups in a purified protein is, however, in harmony with the observations of Wells and Osborne<sup>28</sup> with purified alcohol-soluble proteins from wheat, rye and barley.

In agreement with the results of Bauer and St. Engel, it was found by Kudicke and Sachs<sup>29</sup> that under suitable conditions antimilk serum will, by virtue of the whey proteins, give complement-fixation reactions with blood serum of the same species, but if milk is boiled, the antiserum it engenders will react with milk but not with blood serum. (Evidently this depends on the coagulability of the whey and serum proteins and the incoagulability of casein.) Antiserum for bovine serum will give slight reactions with cow's milk boiled for not over ten minutes. Uhlenhuth and Haendel<sup>30</sup> had previously found that boiled milk gave anaphylactic reactions only in animals sensitized with milk, whereas raw milk caused reactions in animals sensitized with either milk or serum of the same species.

<sup>25</sup> Arch. f. Kinderheilk., 1911, 56, p. 358.

<sup>26</sup> Biochem. Ztschr., 1911, 31, p. 46.

<sup>27</sup> Folia microbiol., 1912, 1, p. 101.

<sup>28</sup> Jour. Infect. Dis., 1913, 12, p. 341.

<sup>29</sup> Ztschr. f. Immunität., 1914, 20, p. 316.

<sup>30</sup> Ibid., 1910, 4, p. 761.

Versell<sup>31</sup> separated casein by adding acetic acid just short of precipitation, and then saturating the milk with CO<sub>2</sub>; the precipitate was washed five times by centrifugation. The whey proteins were not separated from one another. Using the complement-fixation reaction, he found that antiserum for cow's milk reacted with goat's milk, but less strongly than with cow's milk, and more strongly than with human milk; the difference between the caseins of the three species was much less, and most between the wheys. Human milk antiserum gave reactions with human milk diluted to 1:31,250, with cow's milk at 1:1,250, with goat's milk at 1:50. Antiserums for milk or whey gave slight reactions with the serum of the same animal, but anticasein serum did not, as observed by Bauer and St. Engel, and Kudicke and Sachs; also heated casein was still antigenic but heated whey was not. Whey proteins showed the same degree of species specificity as blood serum, but casein showed much less species specificity, being rather in the class of the organ-specific proteins. He found the thermó-resistance of casein is only relative, for it is inactivated if heated sufficiently long or high.

Gay and Robertson<sup>32</sup> found that isolated casein, as well as the "paranuclein" derived from it by peptic digestion, were both distinctly antigenic, one sensitizing against the other. Immunization with casein produced much more active precipitating and complement-fixing antiserums than did paranuclein, and these antiserums also reacted much more strongly with casein than with paranuclein. That is, partial peptic digestion reduced but did not destroy the antigenic power of casein. Completely digested casein was not antigenic. When united with protamine, it retained its antigenic power, the resulting antiserum reacting with either casein or protamine caseinate.<sup>33</sup> These authors believed that the paranuclein synthesized by the "reverse action" of pepsin on the products of peptic digestion of casein is an effective antigen, sensitizing and immunizing (complement fixation) both to itself and the paranuclein produced by partial peptic digestion, and thought that they had thus established the identity of the materials and also that synthesis of a true antigenic protein had been accomplished.<sup>34</sup>

The statement that casein is not an active antigen, made by Dale and Hartley,<sup>35</sup> is supported by the fact that injection of milk itself has been used extensively in nonspecific foreign protein therapy, ordinarily without harmful results, and Müller<sup>36</sup> states that in an extensive experience he has never observed serious symptoms. However, the next article in the same journal, by Lubiner,<sup>37</sup> describes a nearly fatal reaction following intramuscular injection of 10 c.c. of milk into a girl, 8 days after the last of 3 daily injections. Oppenheim<sup>38</sup> also observed a similar case of severe reaction from milk. Of some interest may be the observation that the serum of parturient and lactating women produces a stronger positive Abderhalden reaction with casein than does the serum of men or normal or pregnant women (Kastan<sup>39</sup>). Also we may mention that milk has hemolytic properties, as first shown by Bertarelli.

<sup>31</sup> Ibid., 1915, 24, p. 267.

<sup>32</sup> Jour. Exper. Med., 1912, 16, p. 470.

<sup>33</sup> Gay and Robertson: Ibid., 1912, 16, p. 479.

<sup>34</sup> Ibid., Jour. Biol. Chem., 1912, 12, p. 233.

<sup>35</sup> Biochem. Jour., 1916, 10, p. 431.

<sup>36</sup> Deutsch. med. Wchnschr., 1918, 44, p. 545.

<sup>37</sup> Ibid., p. 547.

<sup>38</sup> Wien. klin. Wchnschr., 1917, 30, p. 1519.

<sup>39</sup> München. med. Wchnschr., 1914, 61, p. 2126.

Pfaundler and Moro found that it contains hemolytic complement. Bauer, Kopf and Sassenhagen observed that this is especially abundant in colostrum, which was corroborated by Schmidt (lit.).<sup>40</sup>

Taken all together, the results obtained by the several observers quoted seem to point definitely to the following conclusions:

1. Biologically, casein is quite as distinct from the whey proteins and the serum proteins as it is chemically.

2. The biologic reactions and chemical composition of caseins from different species of animals show close relationships.

3. The whey proteins as a whole are biologically similar to the soluble serum proteins of the animals from which they are derived. Colostrum contains a larger proportion of proteins of this type.

4. Casein from the milk of an animal of any given species shows a closer biologic relationship to the casein of another species than it does to either the whey proteins or to the serum proteins of its own species; the same is true of the chemical relations.

It is evident, however, that the amount of work done with proteins reasonably well isolated is as yet small, and in need of amplification and control.

#### THE CHEMISTRY OF MILK PROTEINS

In recent years the proteins of milk have received more careful study than previously, and several new facts must receive consideration in biologic work. Applying D. D. Van Slyke's amino nitrogen method, Crowther and Raistrick<sup>41</sup> found that from the chemical standpoint casein, lactoglobulin and lactalbumin were sharply differentiated and distinct proteins, and that they have the same composition whether prepared from colostrum or from milk. Only 0.03% of true globulin was found in milk; it was much more abundant in colostrum. As far as could be determined, the proteins designated as eulactoglobulin and pseudolactoglobulin are chemically identical. Lactoglobulin showed an amino-acid make-up similar to that of globulin from ox-blood, but marked differences were found between lactalbumin and serum albumin. These observations correspond with the results of the immunologic demonstration that casein is distinct from the whey proteins, and that the latter possess features common to the serum proteins of the same species. A series of milkings made twice daily after calving were analyzed, the average composition of the milk from 7 cows showing a steady drop in content of protein between the first and eighth milkings, the percentage of nitrogen present in the milk being shown in table 1. These figures indicate the relatively great abundance of globulin in colostrum, and its rapid reduction within the first 48 hours of lactation.

While caseins from different species do not show demonstrable quantitative or qualitative chemical differences by ordinary methods, a study of the products of racemization by Dakin's method yielded to Dudley and Woodman<sup>42</sup>

<sup>40</sup> Arch. f. Kinderheilk., 1911, 56, p. 342.

<sup>41</sup> Biochem. Jour., 1916, 10, p. 438.

<sup>42</sup> Ibid., 1915, 9, p. 97.

evidence of some structural differences. Caseins from sheep's and cow's milk consist of identical amino-acids, apparently in identical proportions, but when racemized the sheep's casein showed all the tyrosine and most of the lysine unracemized, while both were completely racemized in cow's casein.

Dale and Hartley<sup>33</sup> state that caseins from different species show no clear disparity of antigenic properties, but it is possible that finer quantitative methods will disclose distinct discrepancies, as was found to be the case with the albumins from hen's eggs and duck's eggs. These same authors state that the antigenic properties of casein are relatively feeble.

Osborne and Wakeman<sup>43</sup> found that the protein called lactoglobulin differed much in its solubilities from other globulins, and that it contained 0.24% of phosphorus, in which respect it resembled ovovitellin. In addition they obtained a small quantity of material that seemed to be a protease, although not enough was obtained to permit positive identification. Of particular interest is the discovery that a protein is present in small amount in milk, which is readily soluble in 50-70% alcohol. This alcohol-soluble milk protein in its content of amino-acids is distinctly different in composition from casein.

We, therefore, have chemical evidence that milk contains at least 4 chemically distinguishable proteins: (1) casein, characterized by a

TABLE 1  
PERCENTAGE OF NITROGEN IN MILK

	Total	Casein	Albumin	Globulin	Nonprotein
1st milking.....	2.40	0.75	0.14	1.32	0.19
4th milking.....	2.40	0.51	0.11	0.31	0.04
8th milking.....	0.65	0.46	0.05	0.12	0.02

high content of phosphorus: (2) lactalbumin, a water-soluble protein which contains no phosphorus; (3) lactoglobulin, which contains 0.24% of phosphorus; and (4) the alcohol-soluble protein recently described by Osborne and Wakeman.

#### EXPERIMENTAL PART

Before describing our results we would first call attention to the fact that anaphylaxis experiments should be performed only with animals that have been raised on a known dietary which does not contain the proteins under investigation, and the same should be true of the mothers, since a certain amount of active immunization may result from the food proteins, which can be passively transferred to the fetus. It has been shown by one of us<sup>44</sup> that young guinea-pigs fed on a given protein (egg white or milk) after a short time become sensitized to that protein so that they will give typical reactions when it is injected

<sup>43</sup> Jour. Biol. Chem., 1918, 33, p. 7.

<sup>44</sup> Wells, H. G.: Jour. Infect. Dis., 1911, 9, p. 147.

parenterally. If the feeding is long continued the animals become so immunized after a time that even a sensitizing injection of this protein will not render them hypersensitive, and a second injection has no effect. Guinea-pigs raised largely on oats, therefore, do not give good

TABLE 2  
CASEIN

	Sensitizing Dose, Gm.	Intoxicating Dose, Gm.	Results	Remarks
	Casein	Casein		
1	0.001	0.05	Died, 5 minutes	
2	0.001	0.05	Died, 15 minutes	
3	0.050	0.05	Severe	Note large sensitizing dose
4	0.050	0.05	Severe	
5	0.005	0.001	Slight, doubtful	
6	0.005	0.005	Slight	
7	0.005	0.010	Died, 60 minutes	
8	0.005	0.020	Severe	Nearly died
9	0.005	0.000,1	Died, 2 minutes	Intracardiac
10	0.005	0.000,02	Moderate	Intracardiac
11	0.005	0.000,01	Doubtful	Intracardiac
12	0.005	0.000,01	Slight, moderate	Intracardiac
13	0.001	0.100	0 ?	
14	0.001	0.100	Moderate	
15	0.000,5	0.100	Severe	Nearly died
16	0.000,5	0.100	0	
17	0.000,1	0.100	Slight	
18	0.000,1	0.100	0	
19	0.000,05	0.100	0	
	Casein	Lactalbumin		
20	0.050	0.100	Slight	
21	0.005	0.050	Slight	
22	0.005	0.050	0	
	Lactalbumin	Casein		
23	0.005	0.050	0	
24	0.005	0.050	0	
	Casein	Lactoglobulin		
25	0.005	0.050	0	
26	0.005	0.050	Doubtful	
	Lactoglobulin	Casein		
27	0.005	0.050	0	
28	0.005	0.050	Slight	
	Casein	Alcohol soluble protein		
29	0.001	0.050	Slight	
30	0.001	0.050	0	
31	0.005	0.050	0	
32	0.005	0.050	Moderate	
	Alcohol soluble protein	Casein		
33	0.005	0.050	Moderate	No protection
34	0.005	0.050	Severe	No protection
35	0.005	0.050	0	No protection
36	0.005	0.050	0	No protection
37	0.005	0.050	Died, 10 minutes	
38	0.005	0.050	Slight	
	Casein	Beef serum		
39	0.005	1.0 c c	0	
40	0.005	1.0 c c	0	
	Beef serum	Casein		
41	0.1 c c	0.050	0	
42	0.1 c c	0.050	0	

reactions with oat proteins, although readily sensitized to other, unrelated vegetable proteins; and guinea-pigs raised on corn were found not to serve for reactions with proteins from corn, although readily sensitized to oat proteins. Probably this fact accounts for numerous



failures and irrational results obtained in anaphylaxis work, and must be taken into account, particularly in experiments with proteins such as those of milk, that might be present in the guinea-pig dietary. In these experiments all our guinea-pigs have been raised exclusively on a vegetable diet, as have their mothers.

In determining specificity it is also imperative that every precaution be taken to avoid contamination, especially of the sensitizing doses, with even the smallest possible quantity of the protein that is to be used for the intoxicating dose. As numerous articles discussing anaphylactic specificity do not mention these precautions, leaving the reader uncertain as to the significance of the results described, we state explicitly that in this, as in all our work, such contaminations were rigidly excluded. All glassware, after thorough washing, is kept for at least 24 hours in concentrated sulphuric acid and potassium bichromate; the needles are most thoroughly cleaned and kept in absolute alcohol.

During the course of this work several preparations of various fractions, obtained in the isolation and purification of the milk proteins, were tested by means of the anaphylaxis reaction. The following tables give the results obtained with purified preparations of the 4 known proteins. The terms used are as defined in our previous work.<sup>45</sup> Unless otherwise specified the injections were made by the intraperitoneal route. The interval between sensitizing and intoxicating injections was usually about from 18 to 20 days. The references to protection indicate the protection of a sensitized animal against reinjection with the homologous antigen after previous injection of some heterologous antigen.

The first nineteen experiments recorded in this table deal with the antigenic properties of casein. They show that, on the whole, casein is not so active as some other soluble proteins in producing anaphylactic sensitization and shock, but, on the other hand, its activity is by no means low. By intracardiac injection the minimum lethal dose is apparently between 0.000,02 and 0.00001 gram, but by intraperitoneal injection it requires nearer 0.010 gram to cause death, and 0.020 gram is not always fatal. Presumably this great difference in the efficiency of intracardiac and intraperitoneal injections of casein depends on the readiness with which casein is thrown out of solution, for with the vegetable proteins we have found that their intraperitoneal toxicity for sensitized animals seems to vary directly with their solubility in the body fluids.<sup>46</sup> As compared with its intoxicating power the sensitizing

<sup>45</sup> Wells, H. G., and Osborne, T. B.: *Jour. Infect Dis.*, 1911, 8, p. 88.

<sup>46</sup> Wells, H. G., and Osborne, T. B.: *Jour. Infect Dis.*, 1914, 14, p. 377.

capacity of casein seems to be very low. Table 3 offers a comparison of the anaphylactogenic activity of the milk proteins with that of other proteins as determined by us.

Experiments 20 to 24 show how completely distinct these preparations of lactalbumin and casein are from one another. The same is shown for casein and lactoglobulin by experiments 25 to 28. Experiments 29 to 38 indicate that the alcohol soluble protein is quite distinct from casein, but that our alcohol soluble protein preparation probably contains a trace of casein, since with 5 mg. sensitizing doses 2 of 4 animals gave severe reactions with casein. Since the alcohol soluble protein is obtained from casein by washing the precipitated casein with alcohol, this admixture is to be expected.

TABLE 3  
COMPARATIVE ANAPHYLACTOGENIC ACTIVITY

Protein	Minimum Fatal Sensitizing Dose, Gm.	Minimum Sensi- tizing Dose, Gm.	Minimal Fatal Intoxicating Dose, Intra- peritoneal, Gm.	Minimal Fatal Intoxicating Dose, Intra- cardiac, Gm.
1. Casein.....	0.001	0.0001	0.010	0.0001
2. Lactalbumin.....	0.001	0.001	0.005	0.0001
3. Lactoglobulin.....	0.001	0.000,01	0.010	
4. Alcohol soluble protein.....	0.001	0.0001	0.010	0.0002
5. Egg albumin.....	0.000 001	0.000,000,1	0.0005	0.000,05
6. Egg globulin.....	0.000,001		0.0008	
7. Edestin.....	0.0005	0.000,000,1	0.100	
8. Squash seed globulin.....	0.000,000,5	0.000,000,5	0.010	
9. Brazil nut protease.....			0.0005	
10. Soy bean protease.....			0.002	

Experiments 39 to 42 indicate the absence in beef serum of appreciable amounts of any protein resembling casein. Elsewhere we have reported observations showing that casein will sensitize to milk, and conversely, as has been observed by others.

The first thirteen experiments of this table indicate that lactalbumin is an active anaphylactogenic substance, thus differing from the albumin fraction of horse serum, which according to numerous observers, is very defective in anaphylactogenic properties.<sup>47</sup> It is much less actively anaphylactogenic than egg albumin, but corresponds closely to casein.

Experiments 14 to 25 indicate that lactalbumin and lactoglobulin are entirely distinct proteins, but that our preparations are not completely separated, for while 5 mg. sensitizing doses give little or no crossed reactions, 50 mg. sensitizing doses of either preparation will

<sup>47</sup> Dale and Hartley: *Biochem. Jour.*, 1916, 10, p. 408. See Kato, *Mitt. med. Fak. Univ. Tokio*, 1918, 18, p. 195.

do so. As the globulin and albumin separations merely depend on the fact that the globulin comes down in a neutral saturated solution of magnesium sulphate, while the albumin is precipitated from the filtrate of this precipitation by acidification, it is remarkable to find that the separation can be made so nearly complete. The fact that

TABLE 4  
LACTALBUMIN

	Sensitizing Dose, Gm.	Intoxicating Dose, Gm.	Results	Remarks
	Lactalbumin	Lactalbumin		
1	0.005	0.070	Died, 17 minutes	
2	0.005	0.070	Died, 20 minutes	
3	0.100	0.050	Severe	} Note large sensitization dose
4	0.050	0.050	Died, 30 minutes	
5	0.001	0.050	Died, 20 minutes	
6	0.000,1	0.050	0	
7	0.000,01	0.050	0	
8	0.005	0.030	Died, 35 minutes	
9	0.005	0.020	Moderate	
10	0.005	0.010	Died, 70 minutes	
11	0.005	0.005	Died, 50 minutes	
12	0.005	0.000 1	Died, 20 minutes	Intracardiac
13	0.005	0.000 05	0	Intracardiac
	Lactalbumin	Lactoglobulin		
14	0.005	0.070	0	
15	0.005	0.070	0	} No protection
16	0.005	0.050	0	
17	0.005	0.050	0	
18	0.100	0.050	Severe	} Note large sensitization dose
19	0.050	0.050	Severe	
	Lactoglobulin	Lactalbumin		
20	0.005	0.070	Moderate	Partial protection
21	0.005	0.070	Slight	Partial protection
22	0.050	0.050	Severe	} Note large sensitization dose
23	0.050	0.050	Severe	
24	0.005	0.050	0	No protection
25	0.005	0.050	0	Partial protection
	Lactalbumin	Casein		
26	0.005	0.050	0	
27	0.005	0.050	0	
	Casein	Lactalbumin		
28	0.005	0.050	Slight	
29	0.005	0.050	0	
30	0.050	0.100	Slight	} Note large sensitization dose
	Lactalbumin	Alcohol soluble protein		
31	0.005	0.050	0	
32	0.005	0.050	0	
	Alcohol soluble protein	Lactalbumin		
33	0.005	0.050	0	
34	0.005	0.050	0	
	Lactalbumin	Beef serum		
35	0.005	0.5 c c	0	} No protection
36	0.005	0.5 c c	Slight	
	Beef serum	Lactalbumin		
37	0.1 c c	0.070	0	Partial protection
38	0.5 c c	0.050	0	No protection

we are here dealing with distinctly different proteins is certainly demonstrated. Experiments 26 to 30 show that lactalbumin is a distinct protein, and that it can be separated from casein almost perfectly.

The complete distinction of lactalbumin from the alcohol soluble protein is established by experiments 31 to 34, while the distinction from casein has already been commented on.

That the albumin fraction of cow's milk is distinct from the protein called albumin, or any other protein, of cow serum, is shown by experiments 35 to 38. This agrees with the demonstration by Crowther

TABLE 5  
LACTOGLOBULIN

	Sensitizing Dose, Gm.	Intoxicating Dose, Gm.	Results	Remarks
	Lactoglobulin	Lactoglobulin		
1	0.005	0.070	Died, 24 minutes	
2	0.005	0.070	Died, 10 minutes	
3	0.005	0.050	Died, 25 minutes	
4	0.005	0.020	Slight	
5	0.005	0.015	Severe	Nearly died
6	0.005	0.010	Severe	Died after 8 hours
7	0.005	0.010	Moderate	
8	0.005	0.005	Slight	
9	0.050	0.050	Moderate	} Note large sensitization dose
10	0.050	0.050	Severe	
11	0.005	0.000,1	0	
12	0.005	0.000,2	Doubtful	
13	0.005	0.000,5	Slight	Intracardiac
14	0.001	0.050	Died, 40 minutes	Intracardiac
15	0.000,1	0.050	Severe	
16	0.000,01	0.050	Severe	
17	0.000,001	0.050	Slight	
	Lactalbumin	Lactalbumin		
18	0.005	0.070	Slight	
19	0.005	0.070	Moderate	
20	0.050	0.050	Severe	} Note large sensitization dose
21	0.050	0.050	Severe	
22	0.005	0.050	0	Not protected
23	0.005	0.050	0	Not protected
	Lactalbumin	Lactoglobulin		
24	0.005	0.050	0	Not protected
25	0.005	0.050	0	Not protected
26	0.100	0.050	Severe	} Note large sensitization dose
27	0.050	0.050	Severe	
28	0.005	0.050	0	
29	0.005	0.050	0	
	Lactoglobulin	Casein		
30	0.005	0.050	0	Not protected
31	0.005	0.050	Slight	Not protected
	Casein	Lactoglobulin		
32	0.005	0.050	0	
33	0.005	0.050	Doubtful	
	Lactoglobulin	Alcohol soluble protein		
34	0.005	0.050	0	
35	0.005	0.050	0	
	Alcohol soluble protein	Lactoglobulin		
36	0.005	0.050	0	
37	0.005	0.050	0	
	Lactoglobulin	Beef serum, c c		
38	0.005	0.5	Severe	
39	0.005	0.5	Died, 25 minutes	
40	0.005	1.0	Died, 32 minutes	
41	0.005	1.0	Died, 42 minutes	
42	0.001	0.5	Died, 35 minutes	
43	0.001	0.5	Died, 20 minutes	
	Beef serum, c c	Lactoglobulin		
44	1.0	0.060	Slight	
45	1.0	0.040	Moderate	
46	0.5	0.050	Severe	
47	0.5	0.050	Moderate	
48	0.1	0.050	Died, 25 minutes	
49	0.1	0.050	Severe	

and Raistrick<sup>41</sup> of the chemical distinction between bovine lactalbumin and bovine serum albumin.

TABLE 6  
ALCOHOL SOLUBLE PROTEIN

	Sensitizing Dose, Gm.	Intoxicating Dose, Gm.	Results	Remarks
1	Alcohol soluble protein	Alcohol soluble protein		
2	0.005	0.050	Died, 10 minutes	
3	0.005	0.050	Died, 15 minutes	
4	0.005	0.050	Slight	
5	0.005	0.050	Died, 5 minutes	
6	0.005	0.020	Slight	
7	0.005	0.015	Died, 25 minutes	
8	0.005	0.010	Died, 25 minutes	
9	0.005	0.005	Severe	
10	0.005	0.000,1	Moderate	Intracardiac
11	0.005	0.000,2	Died, 2 minutes	Intracardiac
12	0.001	0.100	Died, 4 minutes	Intracardiac
13	0.000,1	0.100	Died, 40 minutes	
14	0.000,01	0.100	Severe	
15	0.000,001	0.100	0	
16	Alcohol soluble protein	Casein		
17	0.005	0.050	Moderate	
18	0.005	0.050	Severe	
19	0.001	0.050	0	
20	0.005	0.050	Died, 10 minutes	
21	0.005	0.050	Slight	
22	Casein	Alcohol soluble protein		
23	0.005	0.050	Moderate	Not protected
24	0.001	0.050	Died	
25	0.001	0.050	Slight	
26	0.005	0.050	0	
27	0.005	0.050	Moderate	
28	Alcohol soluble protein	Milk		
29	0.003	3 c c	0	Protected
30	0.003	3 c c	0	Protected
31	0.003	2 c c	Died, 20 minutes	
32	0.004	2 c c	Severe	
33	0.004	2 c c	Severe	
34	Milk	Alcohol soluble protein		
35	0.4 c c	0.050	Slight	
36	0.4 c c	0.050	0	
37	0.1 c c	0.050	0	No protection
38	0.2 c c	0.050	Slight	
39	0.2 c c	0.050	Died, 40 minutes	No protection
40	1.0 c c	0.050	0	
41	1.0 c c	0.050	Slight	
42	Alcohol soluble protein	Lactalbumin		
43	0.005	0.050	0	
44	0.005	0.050	0	
45	Lactalbumin	Alcohol soluble protein		
46	0.005	0.050	0	
47	0.005	0.050	0	
48	Alcohol soluble protein	Lactoglobulin		
49	0.005	0.050	0	
50	0.005	0.050	0	
51	Alcohol soluble protein	Gliadin		
52	0.005	0.050	0	Not protected
53	0.005	0.050	0	
54	Gliadin	Alcohol soluble protein		
55	Beef serum	Alcohol soluble protein		
56	0.5 c c	0.050	0	
57	0.5 c c	0.050	0	
58	Alcohol soluble protein	Beef serum		
59	0.005	0.5 c c	0	
60	0.005	0.5 c c	0	

Experiments 1 to 17 show that lactoglobulin is an active anaphylactogen. The experiments with casein and lactalbumin are the same as those in previous tables and have already been discussed. The individuality of lactoglobulin and the alcohol soluble protein of milk is established by experiments 34 to 37. Experiments 38 to 49 show that lactoglobulin differs from the other 3 protein fractions of milk, since even in 1 mg. doses it sensitizes effectively to beef serum, and also intoxicates animals sensitized with small amounts of beef serum, corroborating the results obtained with the complement-fixation reaction by Bauer and St. Engel.<sup>46</sup> We thus find that the earlier observations on the capacity of beef serum and cow's milk to sensitize to each other is due solely to the globulin of the milk. This fact is in gratifying agreement with the chemical observation of Crowther and Raistrick since it indicates that bovine lactoglobulin is chemically identical with the globulin fraction of beef serum, although lactalbumin and serum albumin appear to be chemically different proteins.

The observation of Heuner that colostrum is biologically more closely related to serum than later milk, harmonizes with the high globulin figure obtained in colostrum by Crowther and Raistrick. The fact that colostrum contains large amounts of globulin, that this globulin seems to be identical with serum globulin, and that the serum globulin fraction carries the antibodies of the blood, is in interesting agreement with observations that have been made which indicate that the suckling animal secures important accessions to its antibody defense during the first days of life.<sup>48</sup>

The previously reported observations that cow milk and bovine serum are uncertain in their capacity to sensitize to one another is explained by the presence of a very small amount of globulin in milk, corresponding immunologically to serum globulin, for it has been shown by Julian H. Lewis<sup>49</sup> that a small amount of one antigen injected together with another antigen in excessive amounts, may be completely or partly prevented from manifesting its antigenic activity. This probably explains the fact that in our experiments, 0.1 c.c. of beef serum sensitized more effectively than 1 c.c.

We have discussed, in connection with the other tables, the complete differentiation of alcohol soluble protein of milk from the three other protein fractions. It is to be recalled that this protein has only recently been described by Osborne and Wakeman, and that the application of the anaphylaxis reaction played an important part in the differ-

<sup>46</sup> See Reymann: *Jour. Immunol.*, 1920, 5, p. 227.

<sup>49</sup> *Jour. Infect. Dis.*, 1915, 17, p. 241.

entiation from the other proteins of milk. When large quantities of freshly precipitated casein were extracted at room temperature with 50-70% ethyl alcohol, it was found that the alcohol had dissolved out a considerable quantity of protein. As hitherto the only known alcohol-soluble proteins were of vegetable origin, it was suspected that this material might be either a cleavage product or a derivative of the casein. The anaphylaxis test demonstrated that it was not a proteose or similar cleavage product, since these do not exhibit active anaphylactogenic properties as does this alcohol-soluble protein (exper. 1 to 15), while cross sensitization as well as chemical tests showed that it was not casein. Further chemical study showed that it yielded a different proportion of some of the amino acids than did casein and was, therefore, a newly recognized constituent of milk. Although resembling gliadin in solubility, it is chemically distinct from this protein and in agreement therewith yields negative results with cross sensitization tests with gliadin (exper. 50 to 53). The positive cross experiments with milk indicate that it is a constituent of milk, and not formed by manipulation during isolation (exper. 28 to 41). With such a relative excessive proportion of other proteins present in the milk, it is not to be expected that effective sensitization with milk can be obtained. The amounts of the alcohol soluble protein which give strong sensitization to milk are so small (3 to 4 mg.) that sensitization with admixed casein seems to be excluded, since less than 1 mg. doses of casein sensitize but little.

Experiments 54 to 57 indicate that beef serum does not contain the alcohol-soluble protein of milk.

#### SUMMARY

Cow's milk contains 4 chemically distinct proteins or protein fractions, namely, casein, lactalbumin, lactoglobulin and an alcohol-soluble protein. By means of the anaphylaxis test it can be shown that these 4 proteins are immunologically distinct. This fact furnishes another striking illustration of the dependence of immunologic specificity on chemical composition rather than biologic origin. Of these 4 proteins only one, the globulin, sensitizes to beef serum or causes reactions in animals sensitized to beef serum. This corresponds to the observation of Crowther and Raistrick that lactoglobulin and serum globulin are chemically indistinguishable. That some positive cross sensitizations may be occasionally obtained between cow's milk and beef serum is explained by the fact that the globulin constitutes a very small

part of the milk proteins. Several other protein fractions obtained in studying milk proteins were, according to anaphylaxis tests, not distinct from the 4 known proteins of milk. Our experience with milk proteins, as well as with proteins of other sources, has demonstrated that immunologic methods are a great aid and in many cases indispensable in preparing proteins in a state of purity, and may be used to furnish information concerning chemical relations of proteins from different sources.



## EXPERIMENTAL STREPTOCOCCUS PNEUMONIA AND EMPYEMA. III.

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In previous articles<sup>1</sup> we have described a form of experimental streptococcus empyema which can readily be produced in rabbits by direct injection into the pleural cavity of a very small amount (0.1 to 0.2 cc of a 24-hour broth culture) of a passage strain of streptococcus pyogenes (Holman) originally derived from a case of fatal human empyema. This experimental condition in rabbits offers almost complete analogy to the human condition as regards its progress. It is a process of infection by extension proceeding from the side infected and involving the pericardium and the other pleural cavity. The blood gives positive cultures with no great regularity except after death, and no lesions occur elsewhere in the body. This empyema differs from the human condition in being more acute since it invariably terminates fatally in normal animals by the fifth day on an average.

This experimental empyema in rabbits, moreover, differs from its human analogue in its artificial method of origin. Human streptococcus empyema, which has been so frequent and so fatal following measles, influenza, and broncho-pneumonia in general, apparently originates by extension of the micro-organism, which is its causative agent, downward through the respiratory tract. This hemolytic streptococcus is found in the mouth and tonsils, particularly in those who later acquire bronchopneumonia and empyema. There is further evidence that the human infection proceeds very rapidly. The clinical signs of bronchopneumonia are perhaps not even detected before the patient exhibits evidence of a pleural effusion. It seemed to us desirable, therefore, when we began these studies, to endeavor to repeat experimentally, if possible, what is presumably the entire life history of this form of infection in man, and to furnish light on its exact course. Our earlier experiments in this respect were, as we have already stated, a failure. We were unable to produce the slightest result by inoculating large amounts of streptococcus culture into the trachea. In view of our

Received for publication April 27, 1921.

<sup>1</sup> Jour. Infect. Dis., 1920, 26, p. 265; and 1921, 28, p. 1.

present success in obtaining positive results it is evident that our failures were due to several causes which we are now able to analyze by comparison.

Experimental pneumonia with the streptococcus has been produced in dogs by Wollstein and Meltzer<sup>2</sup> and in a few instances by Graham and Bell. In a series of articles beginning in 1919, Wollstein and Meltzer describe the production of a form of bronchopneumonia in dogs produced by means of intrabronchial insufflation. The infection was in no instance fatal in their first two series of animals, but gave rise, as was evident by sacrificing them, to a characteristic form of bronchopneumonia which we will later describe in more detail, and which differs from the form of pneumonia produced by a similar inoculation of pneumococcus. In a more recent article,<sup>3</sup> these same authors obtained a mortality of 41.5% in 24 dogs by using a strain of streptococcus derived from the same series of human empyema cases as the one with which we have worked. This micro-organism, moreover, invaded the blood stream regularly and produced empyema in 12% of the cases, in this respect differing from the two previous strains of streptococcus employed.

Graham and Bell<sup>4</sup> have likewise obtained lobular pneumonia followed by empyema and pericarditis in 3 of 6 dogs which were given insufflation of from 2 to 5 c c of empyema fluid from a dog that had been inoculated directly into the pleural cavity.

In a recent article, Blake and Cecil<sup>5</sup> have produced lobular pneumonia of an interstitial type in 9 monkeys by intratracheal injection of various doses of streptococcus hemolyticus. Death occurred in 3 animals that had received the larger doses (10 c c), and was accompanied by a serofibrinous pleurisy.

#### PERSONAL EXPERIMENTS

We have now succeeded in producing bronchopneumonia followed by empyema in every one of the 18 rabbits in which we have attempted it, in the following manner: The rabbits are etherized, and by means of a soft rubber catheter pushed down the trachea, amounts of culture varying from 0.01 c c (usually diluted to 2 to 3 c c volume in plain broth) up to 10 c c have been employed. The catheter is pushed down into the larger bronchi as far as it will go and the culture fluid injected

<sup>2</sup> Jour. Exper. Med., 1912, 16, p. 126; *ibid.*, 1913, 18, p. 548.

<sup>3</sup> Proc. Soc. Exper. Biol. and Med., 1918, 16, p. 40.

<sup>4</sup> Amer. Jour. of Med. Sc., 1918, 156, p. 839.

<sup>5</sup> Jour. Exp. Med., 1920, 32, p. 4.

from a pipet and followed by forcing in a small volume of air. These cultures were not only of the passage strain used in the production of empyema by direct inoculation, but have been grown for the purpose of this intrabronchial insufflation in 5% rabbit blood broth. Although larger amounts of 4, 6 and 10 c c apparently produced more rapid death, smaller doses of 1 c c and less have also been almost invariably fatal.

In a series of 18 animals 2 were killed 24 and 48 hours after injection, and the others were allowed to proceed to a fatal termination, which occurred in all but one instance, and that with the smallest dose (0.01 c c), in from 1 to 5 days. In all instances, including those dead in 24 hours, marked exudative pleuritis was present, and in all but 3 of these cases the pleurisy was bilateral. The exudate resembles in all respects the one we have described following intrapleural inoculation, that is to say, it is at first serous, then serofibrinous, then serofibrino-purulent in character, and varies in amount from 5 to 25 c c in each pleural cavity. In all but the earliest one of our series pericarditis was also present.

The lungs in this series of animals are not as compressed as in the series obtained with intrapleural inoculations. They are not found collapsed and atelectatic when the cavity is opened, but are dark red and in patches firm in consistency. They may readily be insufflated through the trachea in certain areas leaving firm airless areas which may be simply congested or are definitely consolidated on section. The distribution of these firm areas is irregular, appears in one or both lungs, and tends in 3 to 5 days to become grayish rather than dark red in appearance.

It is apparently necessary to proceed more or less as we have outlined in order to produce positive results. We have found, for example, that simple intratracheal injections of even large amounts of from 10 to 30 c c of our same passage culture made by means of a hypodermic needle between the cartilages of the trachea, will produce no results beyond a local infection. Four negative experiments of this sort were tried. In one of two instances, we did not succeed in producing pneumonia by means of intrabronchial insufflation of 1 c c of our passage culture grown in plain broth instead of blood broth. In the one instance tried serum broth gave the same positive result as blood broth. Although it is true that there are more organisms present in serum broth or blood broth cultures of the same age, we should attribute positive results under the latter conditions to the medium employed

since we have succeeded with very small amounts of the blood-broth culture. Doses of 0.2, 0.02 and one of two doses of 0.01 cc of a 24 hour blood-broth culture, diluted to 3 cc in plain broth, have given positive results.

Microscopic examination of tissues from these animals shows the following stages in the evolution of this process: In 24 to 48 hours, thrombosis of bronchial blood vessels may be found. Desquamation and destruction of both bronchial and alveolar epithelium is evident. Perivascular and peribronchial edema is present. The alveoli are filled in localized areas, often in well marked peribronchial situation, for the most part by desquamated alveolar epithelial cells, but on the 2d to 4th days polymorphonuclear leukocytes to a large extent replace these epithelial cells. Areas of necrosis appear on the 4th day, and from the 3rd to the 5th day well demarcated infarcts may occur, surrounded by zones of infiltration of red blood cells and leukocytes. The alveoli and bronchioles continue to be involved and filled with serum, fibrin and cells. From the 3d to the 4th day the perivascular and peribronchial edema become definitely infiltrated by mononuclear and polymorphonuclear cells, thus suggesting the interstitial bronchopneumonia described by MacCallum<sup>6</sup> in the human cases.

It should be noted that this appearance agrees with the one given by Wollstein and Meltzer<sup>2</sup> in their original communication, and does not differ notably from the one described by Blake and Cecil in monkeys. The essential points in this process and its specificity may well be contrasted with the parallel studies that we have made in a few animals insufflated by the same method with type 1 pneumococcus.

The work on experimental pneumococcus pneumonia is older and more extensive than that on pneumonia produced by the streptococcus. Disregarding the earlier literature in which the authenticity of cultures would naturally be under suspicion, we find that in recent years pneumonia has been produced by means of the pneumococcus in dogs, rabbits and monkeys. The work of Wadsworth,<sup>7</sup> which seems to have inaugurated this recent phase of investigation, is somewhat indefinite as regards the results produced. At all events, the introduction of the method of intrabronchial insufflation by Lamar and Meltzer<sup>8</sup> led to the first complete study of pneumococcus pneumonia in dogs, followed in the same year by a less complete article by Riesman and Kolmer<sup>9</sup> and

<sup>6</sup> Rockefeller Institute, Monograph No. 10, 1919.

<sup>7</sup> Amer. Jour. of Med. Sc., 1904, 127, p. 851.

<sup>8</sup> Jour. Exper. Med., 1912, 15, p. 133.

<sup>9</sup> Trans. Assn. Amer. Phys., 1912, 27, p. 193

another one by Wollstein and Meltzer<sup>10</sup> in the following year. In 1918 Wadsworth<sup>11</sup> reviewed his own work in this field. Whereas Lamar and Meltzer<sup>8</sup> had a mortality of 16%, Wadsworth obtained no fatal results, and the results of Riesman and Kolmer lie intermediate between the two.

Pneumococcus pneumonia has been studied in the rabbit by Winternitz and Hirschfelder<sup>12</sup> and by Klein and Winternitz,<sup>13</sup> who utilized the method of insufflation described by Lamar and Meltzer and obtained a high mortality (85%). More recently Blake and Cecil<sup>14</sup> have described pneumococcus pneumonia in monkeys in a series of articles dealing with pathogenesis and modes of invasion by this micro-organism.

In all these articles the pneumonia described is definitely of the lobar type, both in dogs and rabbits. Pleurisy when described has usually been localized and without effusion (Lamar and Meltzer;<sup>8</sup> Winternitz and Hirschfelder<sup>12</sup>). In most instances the micro-organism has been found in the blood early in the course of the disease.

As a check on the specificity of streptococcus pneumonia, we have inoculated a small series of rabbits by the same intrabronchial method with a culture of type 1 pneumococcus. Our results are similar to those described by Winternitz and Hirschfelder.<sup>12</sup> The eight animals that were inoculated all died within 3 days, apparently of a septicemia, as the blood cultures were uniformly positive when taken during life. The findings in the lung offer certain interesting contrasts to those following streptococcus inoculation. The consolidation produced was at once more extensive and uniform. One or more lobes were in all cases affected, and the distribution of the consolidation was usually throughout the entire lobe or lobes affected. Certain small areas, however, could usually be insufflated.

In one of the eight instances there was a definite pleuritis with effusion, which is in sharp contrast to the uniform and extensive exudate in the streptococcus cases. In 2 of 8 animals examined within the first 24 hours, a slight serous exudate was found, but in the other 6, which died later, there was no pleurisy with effusion, and in only one was a localized dry pleurisy of limited extent observed. A slight serous pericarditis was present in one of the 8 cases.

<sup>10</sup> Jour. Exper. Med., 1913, 17, p. 353.

<sup>11</sup> Jour. Med. Res., 1918, 39, p. 147.

<sup>12</sup> Jour. Exper. Med., 1913, 17, p. 657.

<sup>13</sup> Ibid., 1915, 21, pp. 304 and 311.

<sup>14</sup> Ibid., 1920, 31, pp. 403, 445 and 499.

The microscopic findings in the involved areas are quite distinct from the lesions produced by the streptococcus. Animals dead during the first day show an extreme congestion of the blood vessels. The alveoli are filled with serum, fibrin, and red blood corpuscles. The alveolar and bronchial epithelium remains relatively intact. On the 2d day definite gray hepatization is evident, many alveoli being packed with polymorphonuclear leukocytes, red blood cells and a few epithelial cells. A limited bronchiolitis appears. In animals dying on the 3d day the vessels remain extremely congested, but most of the alveoli have become clear and contain at most small amounts of fibrin and serum. There is a slight peribronchial and perivascular infiltration of mononuclear cells. In other words, the process is apparently in course of resolution and shows none of the necrotic areas present in the streptococcus pneumonia. Death evidently is due to the septicemia already mentioned, which it should be recalled occurs irregularly in the streptococcus infections.

#### THE ROUTE OF INFECTION IN EXPERIMENTAL STREPTOCOCCUS PNEUMONIA AND EMPYEMA

We are now in a position to consider more attentively the actual course of infection in this experimental empyema, which we have produced by a natural route such as the one it presumably follows in human beings. As we have seen, the introduction of small amounts of our streptococcus culture into the bronchi leads very rapidly to involvement of the lungs in the form of a characteristic broncho or lobular pneumonia which presents many analogies to the human condition, which is further paralleled by the rapid appearance of a pleurisy with effusion. As we have noted, rabbits killed or dying from 24 hours on show a profuse pleural exudate quite different from the negative or localized sequels of pneumococcus infection.

A study of the streptococcus in the tissues by means of the Gram-Weigert stain shows that, whereas the organisms are diffusely distributed in the alveoli and alveolar walls after inoculation, they soon become collected in the perivascular lymphatics, and are found within 24 hours in the pleural exudate on the surface of the lung.

The exact route of extension of the streptococcus in human infections of this type has been studied fully by MacCallum<sup>14</sup> and involves rather fundamental considerations as to the finer anatomy of the lung lymphatics. Miller,<sup>15</sup> on the basis of his study, has concluded that the

<sup>14</sup> *Amer. Jour. of Roentgenology*, 1917, 4, 269; *Amer. Jour. of Anat.*, 1905, IV, 445.

lymphatics of the lung run in two directions, mainly from the septum and the walls of the blood vessels toward the hilum where they empty into the bronchial lymph nodes. In the second place, Miller states that the current to the superficial layer of the lung is directed away from the hilum and toward the pleural network, in evidence of which he demonstrated valves that open in this direction. MacCallum,<sup>6</sup> on the other hand, on the basis of his observations thinks "that in the lymphatic apparatus of the lung the natural current is from the pleural surface toward the hilum of the lung." The route of pleural involvement, then, according to MacCallum, although through the lymphatics, would be against the stream, and is brought about by thrombosis of these lymphatics and propagation of bacterial growth through the thrombus. This route would certainly delay the passage of streptococcus from the alveoli into the pleural cavity appreciably.

The present day interest in these actual routes of infection through the respiratory tract and the lungs to the pleura is again brought out by recent work of Winternitz, Smith and Robinson,<sup>16</sup> which indicates that the pathway of infection from the upper respiratory tract into the lung may be by means of the subepithelial lymphatics of the larger bronchi through the nodes at the hilum of the lung and thence through the lymphatics into the lung tissue rather than directly through the bronchioles and alveoli.

These questions are particularly susceptible of experimental study by means of this type of infection in the rabbit that we have described. In the first place, it would not seem evident from a superficial study that the route of infection suggested by Winternitz, Smith and Robinson is a usual one. As we have already stated, we have found it impossible, as in the past others have done, to produce pneumonia with the streptococcus or the pneumococcus by means of simple intratracheal injection, which would certainly allow for absorption through bronchial lymphatics. We have, moreover, endeavored to produce positive results by inserting the needle under the epithelium of the trachea but with equally negative results. Apparently, the deposition of the streptococcus in the smaller bronchi and alveoli is necessary in order to obtain results. When we came to study more minutely the evolution of our experimental empyema through the lung, we had on hand an interesting series of tissues for comparison. The form of empyema in which we have extensively studied various possibilities of vaccine, serum, and dye

<sup>16</sup> Bull. Johns Hopkins Hosp., 1920, 31, p. 63.

therapy has been produced uniformly by injection of a very small amount of broth culture directly into the pleura. With our test dose the results are invariably positive, and a fatal empyema occurs. The appearance of the lungs in the empyema produced by intrapleural inoculation is, however, quite different from the condition found when the empyema is subsequent to a streptococcus pneumonia following intrabronchial insufflation. Following the intrapleural inoculations, the lungs are found extremely atelectatic and reduced to the smallest possible space as contrasted with rather full noncollapsible lungs in the conditions of pneumonia which we have just described. In the first instance, moreover, the lungs can be expanded by insufflation with air after removal from the chest cavity, even after they have been compressed by the pleural fluid for a number of days. On the other hand, the lungs of the pneumonia rabbits, or at least definite areas in them, are incapable of expansion.

The greatest interest lies in the localization of the bacteria in the routes of infection which we are studying. Whereas in the pneumonia the streptococci are diffusely scattered throughout the lungs in the beginning and later concentrated in the lymphatics and pleura, following the empyema produced by intrapleural inoculation, no micro-organisms are found below the layers of fibrin on the surface of the lung. In a series of animals that died from intrapleural injections at intervals of from 1 to 28 days (the latter a partially immunized rabbit) and in which the sections were studied microscopically, in no instance were any micro-organisms found within the lung tissue itself. Various degrees of compression of the alveoli and a congestion were evident, and in the later stages (10-28 days) connective tissue replaced lung tissue proper for the most part. It is evident, then, that the streptococci extend rapidly from the lungs into the pleura, but not in the opposite direction. There are, of course, mechanical reasons to explain this fact. Principally concerned is the negative intrathoracic pressure and the fact that the lung on which pleural fluid presses does not expand normally. It would seem, however, that if the lymphatics open from the pleura toward the hilum, as MacCallum asserts, that at least in the early stages following intrapleural inoculation, streptococci would be demonstrable in the lung tissue. During the early hours after inoculation the lung continues to expand normally since very little fluid accumulates, as can be shown by the fact that thoracentesis is usually negative in the first 12 hours after injection.



We have undertaken to determine the rapidity with which streptococci pass from the lungs into the pleural cavity in rabbits by means of an artificial respiration chamber. The apparatus which we have independently devised and employed is similar to that recently described by Graham.<sup>17</sup> The lung of a normal rabbit freshly removed from the body is suspended in a vacuum bottle and connected with a negative water pressure apparatus so devised that by opening and closing a pinch cock the pressure may be made to increase or decrease rhythmically. The trachea of the suspended lung is tightly tied about a glass tube which protrudes through a cork to the exterior air. The lung is found to expand and collapse in this chamber to all appearances in a manner similar to that in the body.

If a small amount of fluid is introduced through the cannula into the trachea, an exudate appears rapidly on the surface of the lung, being greatest, according to Graham, during expiration. In our experiments, the negative pressure which was allowed to effect the expansion of the lung did not exceed 6 mm. of mercury.

If care is taken to remove a lung with aseptic precautions and to prepare it for the chamber as described, cultures taken from the surface are found to be sterile. If then, after 1 or 2 expansions of the lung in the chamber, 2 to 5 c c of a broth culture of streptococcus are introduced into the trachea and respiration continued for 2 to 5 minutes, cultures from the surface of either or both lobes give colonies of streptococcus when streaked on blood agar. It would appear, then, from such experiments that the streptococcus may reach the pleural cavity through the lung in a matter of minutes, and a free opening of lymphatics toward the pleura would seem to be indicated.

Further consideration, however, has shown us how erroneous it would be to draw conclusions from an experiment of this sort. As we have indicated, the artificial respiration chamber would seem to reproduce natural conditions with fidelity. Our suspicions were awakened, however, in one of our experiments in which through inadvertence the lung was allowed to expand to an abnormal degree. Was it not possible that even in the carefully watched experiments in which the pressure was measured, we might have ruptured the epithelium of the pleura or in some other manner made the passage of bacteria easier than under normal conditions? It obviously became necessary to kill a series of animals at stated periods after intrabronchial insufflation in order to

<sup>17</sup> Jour. Am. Med. Assn., 1921, 76, p. 784.

determine within certain wide limits how long it takes for the injected bacteria to reach the pleural cavity under natural conditions of infection. We were then surprised to find that cultures from both pleural cavities remain sterile  $\frac{1}{2}$  hour and 6 hours after insufflation. An animal killed 12 hours after insufflation had one lung that was firm and hemorrhagic with a bloody serous exudate measuring a little over 1 c.c. and containing numerous streptococci. It is evident, then, that the involvement of the pleura in our experimental streptococcus pneumonia is a matter of several hours rather than of minutes as seemed demonstrated by the *in vitro* experiments.

#### CONCLUSIONS

Experimental pneumonia may be produced in the rabbit by bronchial insufflation of very small amounts of a passage culture of hemolytic streptococcus. Histologically this pneumonia is lobular in distribution, necrotizing in effect, does not resolve readily, and is characterized by peribronchial and perivascular edema and later infiltration of mononuclear cells (interstitial bronchopneumonia). It is quite different in character from the pneumonia produced by the pneumococcus.

These differences are further marked by the occurrence of pleurisy with effusion, involving by extension both pleural cavities and the pericardium, in the streptococcus infection. On the other hand, septicemia is the rule with the pneumococcus infection but not with the streptococcus. Both forms resulted fatally.

The natural route of infection with the streptococcus seems to be from the alveoli to the pleura, rather than by the lymphatics of the larger bronchi. When injections are made into the pleural cavity, the micro-organisms never penetrate through the pleura into the lung tissue. This would militate against the idea of a lymphatic stream from pleura to hilum.

Experiments with an artificial respiration chamber seemed to prove that the streptococcus passes from the lungs to the surface of the pleura in a few minutes. It is evident, however that conclusions derived from such experiments cannot explain conditions in the living body where it is found that involvement of the pleura takes place in a matter of hours (6 to 12) rather than of minutes.

# METHODS OF ISOLATION AND CULTIVATION OF ANAEROBIC BACTERIA

STUDIES IN BACTERIAL METABOLISM. XLIII

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The isolation of anaerobic bacilli in pure culture and their subsequent development in a state of unquestioned freedom from contamination presents one of the more difficult and complex problems of bacteriologic procedure. Prior to the recognition of the prominent part played by anaerobic bacilli as incitants of gas gangrene and similar conditions associated with wounds of warfare, the study of these organisms as a group was much neglected and the technic of anaerobiotic procedure was incompletely developed. For this reason the most striking characteristic of the anaerobic group has been overlooked, namely, the ability of certain members to grow side by side as "pure mixed cultures" for long continued serial transfers on artificial mediums. It must be admitted, however, that the recognition of contaminants in supposedly pure strains of anaerobic bacilli is much more difficult of accomplishment than would appear at first sight. This is due in part to the fact that available descriptions of the anaerobic organisms are for the most part incomplete or inaccurate, both culturally and with reference to morphology and sporulation. Furthermore, the widespread distribution of members of this group in nature provides abundant opportunity for the symbiotic association of unlike strains whose combined activities are mutually advantageous. It is not improbable that the effect of reduced oxygen tension, which is a requirement for anaerobic growth, may in itself have been a potent environmental factor in promoting symbiotic relationships among the members of the group of anaerobic bacilli, although aerobic organisms, such as *Micrococcus ovalis*, are not uncommon contaminants of anaerobic cultures.

Theories and even controversies have arisen from studies on cultures of anaerobic bacilli containing unrecognized and therefore unsus-

pected contaminants. A striking, although not vital, instance is that vigorous discussion which has centered around the conception of "putrefaction," as a bacterial process. A small group of investigators

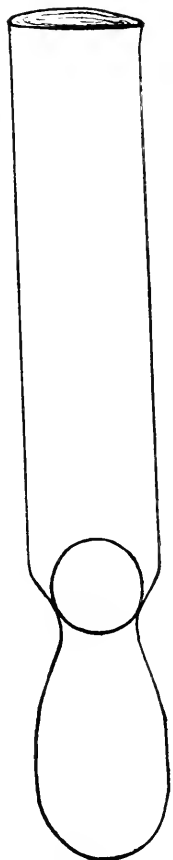


Fig. 1.—Test tube used in experiment performed according to method of Hall.

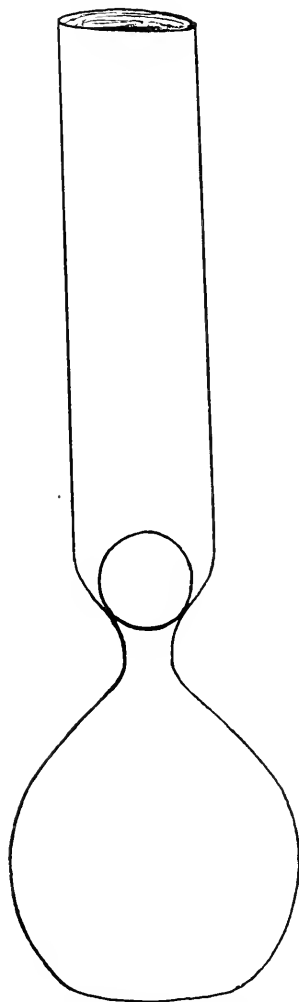


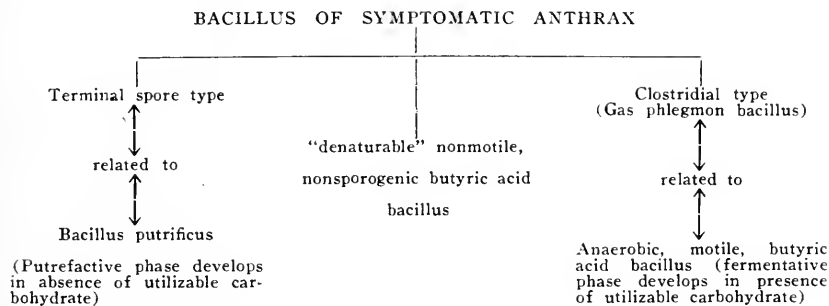
Fig. 2.—Modification of test tube in fig. 1.

have maintained that putrefaction is incited only by anaerobic bacteria, of which *Bacillus putrificus*,<sup>1</sup> the bacillus of malignant edema, and of

<sup>1</sup> Bienstock: *Ztschr. f. klin. Med.*, 1884, 3, p. 1; *Arch. f. Hyg.*, 1899, 36, p. 335; 1901, 39, p. 335. Rettger: *Jour. Biol. Chem.*, 1906-1907, 2, p. 71; 1908, 4, p. 15. Rettger and Newell, *ibid.*, 1912, 13, p. 341.

symptomatic anthrax are regarded as the principal, if not the sole, etiologic types. Putrefaction has been defined by these observers as a decomposition of protein or protein derivatives by these organisms, resulting in the formation of certain foul smelling products. In this respect the process seems to be somewhat similar chemically to the process called "Fäulnis" by the Germans. It is now believed that the bacillus of malignant edema (*Vibrio septique*), the bacillus of symptomatic anthrax, and even *Bacillus putrificus*,<sup>2</sup> freed from contaminating organisms, are distinguished by their feeble action on protein derivatives. *Bacillus tetani* and *Bacillus botulinus* were also formerly regarded as strong proteolytic organisms, but subsequent investigations have shown that powerfully toxicogenic strains, which are practically devoid of proteolytic powers, may be isolated from supposedly pure cultures of these bacteria.

A striking instance of the effect which unrecognized anaerobic symbiosis may have on current bacteriologic thought is that remarkable "dimorphism" of the bacillus of symptomatic anthrax, studied by Grassberger and Schattenfroh, and summarized in Sittler's monograph on intestinal bacteria.<sup>3</sup> The bacillus of symptomatic anthrax, according to these investigations, is said to undergo the following series of transformations as the environmental conditions to which the organism is subjected are changed.<sup>4</sup>



It is now believed that this apparent transmigration of vital energy through a series of nodes of relative cultural stability was due to the

<sup>2</sup> Medical Research Committee: Report on the Anaerobic Infections of Wounds, and the Bacteriological and Serological Problems Arising Therefrom. Special Report Series No. 39, 1919, London. Robertson, Muriel: Brit. Med. Jour., 1918, 1, p. 583. Meyer, K. F.: Jour. Infect. Dis., 1915, 17, p. 458.

<sup>3</sup> Sittler: Die Wichtigsten Bakterientypen der Darmflora beim Säugling, 1909, p. 42.

<sup>4</sup> Chiefly by the addition or elimination of certain nutritional energy compounds.

impurity of the original cultures. Such being the case, it is readily comprehended that from the original mixture one or another of the contaminants developed greatly in excess of the remaining bacilli, as the conditions created in the cultural mediums accelerated or retarded the respective symbiotes, thus suggesting superficially that such a "dimorphism" actually existed. Still another instance of so-called anaerobic transformation is reported by Rosenthal.<sup>5</sup> He believed it was possible to transform the bacillus of Achalmé (*B. welchii* *B. perfringens*) into the enterococcus of Thiercelin (*Micrococcus ovalis*), through a process of acclimatization to exposure to oxygen, until the culture became fully tolerant to the air. When the culture became fully tolerant to the air, the anaerobic bacillus lost its bacillary morphology and characteristics and assumed those of the enterococcus. Here, again, the original culture contained both the Welch bacillus and enterococcus as unrecognized symbiotes. *B. welchii* is now recognized as one of the anaerobic group which is commonly associated with certain putrefactive anaerobes, as *Bacillus sporogenes*, or members of the plectridial anaerobic group, and with *Micrococcus ovalis*. Cultures of the Welch bacillus of undoubted purity, however, never exhibit noteworthy variance in morphology, cultural or serologic reactions.

These examples of unrecognized symbiotism, which represent common experience in the past, are quoted as illustrative of the difficulty of obtaining and maintaining "bacteriologically pure" strains of anaerobic organisms. In defense of those investigators who have recorded these observations, which are at variance with current ideas of specific stability of bacterial types, it may be said that the plating methods used by them, so successful in separating aerobic and facultatively anaerobic organisms from one another, are wholly unreliable as a means for isolating strictly anaerobic bacilli in pure culture. The underlying reason for this marked difference in behavior between the two groups or organisms is at present not satisfactorily determined.

Bacteriologists are now in accord in recognizing that the isolation of single organisms by the Barber method<sup>6</sup> is the most satisfactory basis for the creation of strains of undoubted purity. The method of Barber is highly successful in the study of aerobic and facultatively anaerobic organisms. When it is applied to the anaerobic bacilli, however, the results have been disappointing. This is apparently not

<sup>5</sup> L'aérobisation des microbes anaérobies, 1908.

<sup>6</sup> Kansas Univ. Sc. Bull., 1907, 4, p. 3.

due to an inability to isolate single cells, but rather to the almost uniformly barren results of attempts to secure growths from the primary isolation.

The difficulty is explained in part at least by the unavoidable exposure of the anaerobic vegetative cells to the oxygen of the air. It is well known that the exclusion of more than minimal traces of oxygen from culture mediums is essential for the development of anaerobes, particularly in the earlier hours of growth; hence, one factor of paramount importance for single cell isolations would appear to be the exclusion of air from the material under observation. This may be accomplished either by the use of somewhat cumbersome apparatus during the process of isolation, or it may be accomplished indirectly by taking advantage of the fact that nearly all anaerobic bacilli form spores under suitable conditions; these spores do not appear to be influenced markedly by exposure to the atmosphere, although of course they fail to germinate unless air is excluded.

The first step, therefore, in an attempt to secure pure cultures of anaerobes by the Barber method would appear to be the isolation of single spores from sporulated cultures, previously heated to 80 C. for 10 minutes to kill all vegetative cells. This has been proved to be feasible, and the percentage of successful subcultures thus obtained was found to be materially in excess of that obtained when vegetative cells were isolated in place of spores. The second step which has proved to be of advantage is to place the medium containing the single spore in a vacuum oven,<sup>7</sup> and incubate for a few hours at body temperature<sup>8</sup> in a high vacuum. The combination of spore isolation, and incubation in a high vacuum, has raised the percentage of successful isolations from 1 or 2% to 35%, on the average.

The Barber pipet, as originally described, presents some difficulties of manufacture to the unskilled glass blower, particularly with reference to securing a capillary end of proper size at exactly a right angle to the main portion of the tube. A modification has been substituted which seems to fulfil all the requirements of the apparatus, and in addition to require no degree of precision in its preparation. The pipet, as finally modified, consists of a bent glass tube set in and moved by the regular Barber mechanical pipet holder. The bent end is curved at a right angle in such a manner as to present the lumen of the

<sup>7</sup> The De Freas vacuum oven is well adapted for this purpose.

<sup>8</sup> *Bacillus botulinus* should be grown at 30 instead of at 38 C.

tube in the optical axis of the microscope. A small plug of modeling clay (or a small rubber stopper with a minute central hole) is used to close this end. Sterile capillary tubes of appropriate diameter and length<sup>9</sup> may be inserted in the clay plug (or rubber stopper) so as to move upward by manipulation of the vertical rack and pinions of the mechanical pipet holder to a point directly under the center of the lens of the microscope. The adjustment of such a capillary tube by the pipet holder in such a manner that the open end of the former is clearly visible through the microscope is a comparatively simple technical procedure, readily acquired by practice. The sterile capillary tube is adjusted so that it moves directly upward in the optical axis of the microscope; then it is lowered to a point well below the level of the stage, and a coverglass, previously sterilized, and infected with a drop of glycerine or gelatin bouillon,<sup>10</sup> containing spores, is suspended drop downward, on a shallow, four-sided frame moved by a mechanical stage. The bacterial spores are viewed practically as a "hanging drop" preparation in the usual manner. Any portion of the drop may be brought into the microscopic field by suitable movements of the rectilinear motion of the mechanical stage.

The process of isolating a spore is simple. The spore is located in the preparation, and the surrounding medium is searched for other spores by suitable manipulation of the mechanical stage. If the spore is well isolated, it is again brought into the center of the microscopic field, and the capillary pipet is brought into contact with the under side of the drop at precisely the spot where the spore is located. Capillarity pulls a small amount of medium, including the spore, into the pipet, which is then lowered. The capillary tube is broken off above the clay plug and below the drop of culture medium, with sterile forceps, and the contained spore is transferred to a tube of suitable medium,<sup>11</sup> which is placed in the vacuum oven at 37° C. When the requisite number of tubes containing spores are obtained, the vacuum is created, which draws the minute bubble of air out of each capillary tube, and the medium into it. Each spore is thus localized in a nutrient medium free from oxygen and advantageous for development.

<sup>9</sup> Capillary tubes are drawn out from ordinary glass tubing to the proper degree of fineness, cut into inch lengths, and sterilized in Petri dishes at 180° C. for one hour.

<sup>10</sup> The gelatin or glycerol is added to increase the viscosity of the medium somewhat, thus preventing too free a flow of fluid into the capillary pipet.

<sup>11</sup> It is essential that the medium be heated for some time at the temperature of boiling water, preferably in the Arnold sterilizer, to remove all air. It is then cooled rapidly and inoculated.



The removal of a capillary tube from the holder and the adjustment of a fresh tube in the optical axis of the microscope is the work of less than two minutes. Danger from contamination appears to be minimal, judging from the results of several hundred isolations. When the apparatus is set up, contamination from the stage is guarded against by the protection afforded by the box containing the cover glass; the pipet is long enough to project nearly an inch above the plug of clay which forms the attachment to the pipet holder. In a quiet room, bacteria practically never have become attached to the sides of the capillary pipet.

Unfortunately, the isolation of single cells or single spores does not in itself guarantee pure cultures. Many years ago Theobald Smith<sup>12</sup> pointed out the difficulties encountered in sterilizing milk for bacteriologic purposes, and this difficulty is increased materially in handling mediums containing finely comminuted bits of meat, or other insoluble protein. Milk is a satisfactory fluid medium for use without special arrangements of a mechanical or chemical nature to exclude oxygen, provided the cream is left intact.

It has been the practice in this laboratory to utilize the medium of Von Hibler,<sup>13</sup> the meat mediums of Miss Robertson,<sup>14</sup> and of Holman,<sup>15</sup> and of mixtures of liver and brain for the cultivation of anaerobic bacilli. The medium is dispensed in small tubes, somewhat less than a centimeter in diameter, using a fairly deep column of nutrient material. Such mediums are kept in the autoclave for 2 hours at 20 pounds' pressure, then incubated for 2 days at 37° C., and resterilized for an hour. Experience has shown that this prolonged exposure to heat and pressure kills adventitious spores. The precaution of placing the tubes loosely in the autoclave to permit free circulation of superheated steam is conducive to the success of the operation.

The anaerobes appear to grow well in the meat or brain mediums just described, without additional precautions to exclude the air, provided inoculation is practiced immediately after heating and rapid cooling. This method, however, is wholly inadequate to secure development in peptone nutrient broth cultures.

For peptone mediums not containing meat or other protein reinforcement, some additional factor is required to induce growth. The simplest, which has stood the test of practical experience, is that of

<sup>12</sup> Jour. Exper. Med., 1898, 3, p. 47.

<sup>13</sup> Untersuchungen über die pathogenen Anaëroben, 1908.

<sup>14</sup> Jour. Path. & Bacteriol., 1915-1916, 20, p. 27.

<sup>15</sup> Jour. Bacteriol., 1919, 4, p. 142.

Hall.<sup>16</sup> A test tube is constricted as shown in the illustration (Fig. 1) and sufficient medium is placed therein to cover the constriction with a layer at least 3 or 4 cm. deep. A porcelain ball or a glass marble, nearly the diameter of the tube, is dropped in and the entire apparatus is sterilized in the customary manner. The presence of the ball effectually maintains a condition of anaerobiosis, of sufficient degree to permit the development of almost all anaerobic bacilli, even in sugar-free nutrient broth.<sup>17</sup>

A modification of this apparatus, containing somewhat more than 100 c.c. up to the constriction, has been found very useful in studying the metabolism of anaerobes, when considerable volumes of fluid are required for the several analyses to be made from each culture (Fig. 2).

The flasks are preferably cooled rapidly after preparation (although this is not absolutely necessary), and inoculation is readily accomplished by tilting the flask sideways until the ball rolls out of its seat to the side of the tube. A passage is thereby opened to the underlying medium, into which a drop of active culture practically invariably induces growth. The flask is righted after inoculation, and the ball again seated on the constriction, thus sealing the underlying fluid from the oxygen of the air. Of course the flasks are plugged in the ordinary manner with cotton.

The escape of gas incidental to development is readily accomplished. When the pressure of gas within the flask increases somewhat, the ball automatically lifts, the gas escapes and the ball reseats itself. It is necessary to heat and to cool the flasks slowly in order to prevent violent boiling at the constriction. If this happens, the pressure within the flask may become sufficient to expel some of the contents violently and wet the cotton plug.

These methods of isolation and of cultivation of anaerobic bacilli have been more successful than others thus far studied, but it is freely admitted that the method of isolation of single cells or single spores by the Barber method is time consuming and tedious; the results are much less constant when working with anaerobic bacilli than when isolating aerobes. A method which will combine reasonable speed with unqualified accuracy is urgently needed. The claim for recognition that the single cell method presents, however, is the possibility of obtaining results of undoubted integrity, and this factor alone far outweighs any less trustworthy means in the present state of information about the group of anaerobic bacteria.

<sup>16</sup> University of California Publications in Pathology, 1915, 2, p. 147.

<sup>17</sup> A layer of paraffin oil may be placed above the medium to restrict evaporation; it does not, however, of itself maintain anaerobiosis.

# THE PRODUCTION OF AMMONIA AND CARBON DIOXIDE BY STREPTOCOCCI

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The physiology of the streptococci has been extensively studied, largely, however, in relation to the fermentation of carbohydrates and related substances. As a result of such work, many attempts to classify the streptococci have been made. Some investigators based their classifications entirely on fermentation tests, while others relied on the action of the streptococci on blood corpuscles, and for this purpose blood-agar plates have been found valuable.

It seems to us that neither the fermentations alone nor the reactions on blood corpuscles can give sufficient data to enable one to classify the streptococci with any degree of accuracy. Probably the best means of classifying at present is on the basis of both the fermentations and the blood reactions.

In recent years numerous attempts have been made to separate the streptococci by means of agglutination and the complement-fixation tests. With the latter test results have been obtained which give promise of being valuable, as they may point to the homogeneity of certain groups. While immunologic reactions offer an attractive field for study of streptococci, more intensive work along the line of the physiology of these organisms may also give valuable results. If we are to be satisfied to limit our physiologic studies to the determination of titratable acidity in various carbohydrates, alcohols, and glucosides, perhaps one may say that further studies along this line will be fruitless. Certainly the literature shows that this field has been thoroughly cultivated, and the knowledge secured has undeniably paid for the work. But many old fields yield new and valuable products by improved methods of cultivation; so with the streptococci. The discarding of titration methods for acidity and the substitution of the determination of the hydrogen-ion concentration has brought out new and apparently fundamental differences.

The difference in the final hydrogen-ion concentration between streptococci as pointed out by one of us, Ayers<sup>1</sup> is an example of the

Received for publication May 9, 1921.

<sup>1</sup> Jour. Bacteriol., 1916, 1, p. 84.

advantage of improved methods which often make small differences significant. Whether the final hydrogen-ion concentration in a fermentation represents the limiting  $P_H$  for the organism and causes its growth to cease or whether something else arrests growth and the final  $P_H$  is merely incidental, under definite conditions and with the proper medium these differences are constant. The final  $P_H$  of cultures can be varied, and this fact may present opportunities which have been overlooked by the investigators who apparently see nothing in the hydrogen-ion measurement. Although there is no explanation for this difference in  $P_H$  at present, it does not prevent the use of this difference in helping separate the streptococci. The value of the difference in final hydrogen-ion concentration has been shown by Avery and Cullen,<sup>2</sup> who found small but constant differences between hemolytic streptococci from human and bovine sources. It is, perhaps, to such small differences in the physiology of the streptococci that we must look as a means of recognizing different varieties more accurately.

Ammonia production as a measure of proteolysis has been used frequently in studying bacteria. Generally, however, it is the degree of proteolysis which has been used in classification and ordinarily proteolytic organisms are classed on the basis of the visible liquefaction of gelatin or the digestion of casein or fibrin. There is no reason to suppose, however, that bacteria which show no visible signs of the degradation of proteins may not form definite and characteristic amounts of ammonia under controlled conditions.

Proteolysis by the streptococci has been observed by Jensen,<sup>3</sup> Barthel,<sup>4</sup> Itano<sup>5</sup> and Tisser,<sup>6</sup> while ammonia production, in small amounts, has been noted by Kendall<sup>7</sup> and his associates. Numerous investigators have observed alkaline reactions in fermentation tests, yet little attention has been given to this subject.

It was observed by Evans<sup>8</sup> that certain types of streptococci in a carbohydrate-free yeast-peptone medium changed the  $P_H$  from 6.0 to about 6.8, and it was believed that this indicated a vigorous proteolysis. In this connection it must be pointed out, however, that it is unsafe to assume that the alkaline change in reaction is due to proteolysis for.

<sup>2</sup> Jour. Exper. Med., 1919, 29, p. 215.

<sup>3</sup> Landw. Jahrb. Schweiz., 1904, 18, p. 319.

<sup>4</sup> Centralbl. f. Bacteriol., 11, 1915, p. 76.

<sup>5</sup> Bull. Mass. Agr. Exp. Station, 1916, No. 167.

<sup>6</sup> Compt. rend. Soc. Biol., 1920, 83, p. 110 and p. 127.

<sup>7</sup> Jour. Biol. Chem., 1912, p. 215 and p. 219.

<sup>8</sup> Jour. Agric. Research, 1918, 13, p. 235.

as has been shown by Ayers, Rupp and Johnson,<sup>9</sup> there may be a fermentation of the salts of organic acids which results in the production of alkaline carbonates. It is difficult to prepare a medium in which streptococci will grow which does not contain organic-acid salts. Generally speaking, it is unsafe to make definite assumptions from the alkaline change in reaction, and as the detection of ammonia is a simple matter, as will be shown later, such determinations should be made.

Besides ammonia production, we wish to call attention to the fact that carbon-dioxide production by the streptococci has been largely overlooked. Freudenreich<sup>10</sup> was apparently the first to describe streptococci which produced this gas and Jensen<sup>11</sup> also reports the presence of gas-forming streptococci in his collection of cultures. Evans<sup>8</sup> found similar types in cheese. We venture to say that the production of carbon dioxide by the streptococci has been overlooked, largely because of the methods by which the gas is usually determined in bacteriologic work.

In this paper we shall discuss ammonia and carbon dioxide production by the streptococci and will show that although in some cases the amount is small, it is quite constant. The use of the presence or absence of these products in cultures as a means for separating streptococci will also be pointed out.

#### AMMONIA AND CARBON-DIOXIDE PRODUCTION FROM PEPTONE

While studying the growth of streptococci in 4% peptone solutions, it was thought worth while to make determinations of the ammonia formed. For this work 2 cultures were selected, one representing a typical udder type and the other a fecal type from cow feces. In order to obtain a heavy growth 0.2% dextrose was added to the 4% bacto-peptone solution and also 0.5% potassium dibasic phosphate to provide sufficient buffer. The reaction was adjusted to  $P_H$  7.5. Ammonia determinations by Folin's method, after 7 days' incubation at 37 C., showed that the udder culture produced a small amount of ammonia while the fecal culture showed only a trace. The experiment was repeated, and the same results obtained. Other cultures were examined, and the results indicated that small but quite constant amounts of ammonia were produced by certain streptococci and not by others.

<sup>9</sup> U. S. Dept. of Agric., 1919, Bull. No. 782.

<sup>10</sup> *Zentralbl. f. Bakteriol.*, 1897, 11, 3, p. 47.

<sup>11</sup> *Lactic Acid Bacteria*, 1919.

Some of the medium used for ammonia production was also placed in Eldredge tubes which were inoculated with the udder and fecal types of streptococci, and determinations were made of the amounts of carbon dioxide produced. The Eldredge tube described by Eldredge and Rogers<sup>12</sup> consists of two inverted "T" tubes placed side by side and connected by a third tube. The culture medium is placed on one side and cotton plugs placed in the ends of the inverted "T" tubes. At the time of inoculation a definite amount of N/10 barium hydroxide is placed in the other side of the fermentation tube, and the two open ends are plugged with rubber stoppers which are placed over the cotton plugs. The carbon dioxide formed and released from the medium is measured by the amount of N/10 barium hydrate neutralized by the carbon dioxide as shown by titration with N/10 oxalic acid. Since 1 c c of N/10 Ba (OH)<sub>2</sub> is equivalent to 1.261 c c of CO<sub>2</sub> at 25 C. and 760 mm. pressure, it is a simple matter to calculate the amount of CO<sub>2</sub> produced.

Preliminary experiments showed that cultures which produced ammonia also produced carbon dioxide and those giving no ammonia showed no carbon dioxide. These results indicated that there might be a correlation between NH<sub>3</sub> and CO<sub>2</sub> production.

Although the amounts of ammonia and carbon dioxide produced were small, the results were so constant that similar determinations were made in the following medium in order to ascertain whether the same results could be obtained. The medium consisted of infusion broth with 4% bacto-peptone, the reaction being adjusted to P<sub>H</sub> 7.5. It was found that the amounts of ammonia and carbon dioxide formed agreed closely with those obtained in the peptone-dextrose medium. In general, however, the infusion-peptone medium gave more constant amounts of CO<sub>2</sub> than did the peptone-dextrose medium.

The results obtained from these experiments led to a study of the conditions under which ammonia and carbon dioxide were produced. As the preliminary experiments indicated that CO<sub>2</sub> did not come from the dextrose it seemed possible that it might come from the peptone. If this were true, then increasing the peptone in the medium would increase the amount of CO<sub>2</sub> produced, and further, as CO<sub>2</sub> production seemed to correlate with NH<sub>3</sub> formation, it seemed probable that the ammonia might also be increased.

From the results in table 1, it is evident that such was the case. Infusion broth containing amounts of bacto-peptone ranging from 1 to

<sup>12</sup> *Centralbl. f. Bakteriol.*, 1914, 11, 40, p. 5.

10% was used. The reaction of the medium was adjusted to  $P_H$  7.2. Two cultures were studied, 16 H-1 being a streptococcus from the udder of the cow and F 2-7 representing a typical fecal type from cow feces. The cultures were incubated for 7 days at 37 C., and the amounts of  $NH_3$  and  $CO_2$  produced were then determined. It will be noted that as the peptone was increased there was a constant increase in ammonia from culture 16 H-1, and there was a similar increase in the amount of carbon dioxide. However, the fecal type of streptococcus, culture F 2-7, showed little increase in  $NH_3$  with the increase in peptone and no increase in  $CO_2$ . These results indicated that the ammonia and carbon dioxide came from the peptone and were quite closely correlated. It is obvious from table 1 that 4% bacto-peptone is sufficient to show distinct differences between the two cultures in their ability to form ammonia and carbon dioxide. The udder culture 16 H-1 showed 19.47 mg. of ammonia-nitrogen per 100 c c in excess of the control, while the fecal culture F 2-7 showed only a trace. There were 5.3 c c of  $CO_2$  produced by 16 H-1 and 0.3 c c by F 2-7. The latter amount is within the limits of error of the method and may be considered negative. It should be understood that throughout this paper the amount of  $CO_2$  is expressed as cubic centimeters of gas from 15 c c of medium.

TABLE 1  
EFFECT OF PEPTONE ON AMMONIA AND CARBON-DIOXIDE PRODUCTION

Bacto-peptone, Percentage	NH <sub>3</sub> -N. Mg. per 100 C c; Excess over Control		CO <sub>2</sub> . C c of Gas from 15 C c of Medium; Excess over Control	
	Culture 16 H-1 Mg.	Culture F 2-7 Mg.	Culture 16 H-1 C c	Culture F 2-7 C c
1	8.12	0.28	2.0	0.2
4	19.47	0.28	5.3	0.3
6	34.46	1.82	7.1	0.5
8	43.85	2.80	8.4	0.3
10	51.00	2.66	10.5	0.2

The incubation period used in the experiments was 7 days, but to reach the maximum ammonia production this length of time was not necessary. This is evident from table 2 in which it will be seen that the ammonia reached its maximum in 24 hours. There was, however, an increase in  $CO_2$  up to the seventh day, but after 24 hours' incubation sufficient  $CO_2$  was found to give a positive test for evidence of its production by the culture.

Since the ammonia and carbon-dioxide production under discussion was dependent on the peptone content of the medium, it was necessary to know whether or not different makes of peptone would give similar

results. Ammonia productions from 4 makes of peptone were compared, infusion broth being used as a basis for the medium. The reaction of each medium was adjusted to  $\text{pH}$  7.5. Both 1 and 4% peptone were used and inoculated with cultures 16 H-1 and F 2-7 and incubated 7 days at 30 C. It will be remembered that 16 H-1

TABLE 2  
RATE OF AMMONIA AND CARBON-DIOXIDE PRODUCTION FROM PEPTONE

Days	NH <sub>3</sub> -N Mg. per 100 C c; Excess over Control Mg.	CO <sub>2</sub> C c Gas from 15 C c of Medium; Excess over Control Mg.
1	22.41	2.6
2	22.27	4.3
3	21.57	4.3
4	22.27	5.0
5	21.15	4.8
6	21.71	5.0
7	21.85	5.3

produced ammonia in 4% bacto-peptone-infusion broth while F 2-7 did not. The results in table 3 are of considerable interest since they show that ammonia was derived from some ingredient that probably was not present in the same amount in all makes of peptone. For example, culture 16 H-1 showed 23.67 mg. of ammonia-nitrogen per 100 c c of medium when bacto-peptone was used, 11.91 mg. with Parke-Davis' peptone, 6.30 mg. from Fairchild's peptone and only 3.92 mg. per 100 c c with Merck's peptone ("made from albumin"). These

TABLE 3  
AMMONIA PRODUCTION FROM DIFFERENT PEPTONES

Infusion Broth with Different Peptones	Culture 16 H-1		Culture F 2-7	
	NH <sub>3</sub> -N, Mg. per 100 C c; Excess over Control		NH <sub>3</sub> -N, Mg. per 100 C c; Excess over Control	
	1% Peptone	4% Peptone	1% Peptone	4% Peptone
Bacto.....	8.54	23.67	0.14	0.56
Parke Davis.....	4.34	11.91	0.28	1.26
Fairchild.....	3.64	6.30	0.28	0.14
Merck (from albumin).....	2.10	3.92	0.56	2.80

figures represent the amount of ammonia-nitrogen in excess of the control. Culture F 2-7 showed practically no ammonia with any of the peptones. It is at once evident that whatever is the ingredient of peptone which yields the ammonia, it is not present to the same extent in all peptones and it is necessary, therefore, that the test always be run with a peptone which is known to contain the necessary substance.



It must be remembered that ammonia production by streptococci is small but quite constant in amount; therefore the peptone must be used which gives the largest quantity. Several lots of bacto-peptone were examined to see whether variable results would be obtained. While there was some variation in the quantity of ammonia and carbon dioxide formed, the differences were of a minor nature. The substance in peptone from which the ammonia and carbon dioxide are derived is apparently not present in the controllable ingredient which is added to bacto-peptone.<sup>13</sup> Some of the other peptones from which smaller amounts of ammonia were produced reduced the figures to such small amounts as to make real differences in ammonia production by different streptococci a questionable matter.

What has been said of ammonia production is true also of carbon-dioxide production. Table 4 shows that the amount of CO<sub>2</sub> produced by culture 16 H-1 was extremely variable when different peptones were used. As with ammonia, the largest CO<sub>2</sub> production was observed when bacto-peptone was used. Here again NH<sub>3</sub> and CO<sub>2</sub> production seemed to be correlated for, as may be observed, the peptones may be listed in the same order either according to ammonia or carbon-dioxide formation.

TABLE 4  
CARBON DIOXIDE PRODUCTION FROM DIFFERENT PEPTONES

Culture	Infusion Broth with 4% Peptone				
	Bacto-peptone, C c	Parke-Davis, C c	Fairchild, C c	Witte, C c	Merck, from Albumin, C c
16 H-1	5.2	2.6	1.9	1.4	0.1
F 2-7	0.7	0.3	0.4	0.3	0.6

In the hope of being able to throw some light on the probable source of the ammonia, numerous other substances were tried to ascertain whether they contained the ammonia-yielding ingredient. Among these were aminoids made from egg albumin, beef, casein, fibrin and gelatin. It was thought, since these aminoids were made from quite different materials, that the difference in the kinds of amino acids likely to be present might give some clue as to the ammonia yielding fraction.

Infusion broth was used as a basis for the mediums to which was added 0.5%, 1.0% and 2.0% of the various aminoids. The reaction in all cases was adjusted to P<sub>H</sub> 7.5. These mediums were inoculated

<sup>13</sup> We wish to express our thanks to Dr. J. W. M. Bunker of the Digestive Ferments Company for his kindness in supplying samples of peptone containing increased amounts of a controllable ingredient.

with an ammonia-producing streptococcus 16 H-1 and also with F 2-7, which produced no ammonia. The latter culture, as shown in table 5, produced practically no ammonia, while the results obtained with culture 16 H-1 in 2% aminoid need only be discussed. The 0.5 and 1.0% solutions of the aminoids did not in any case show a sufficient amount of ammonia for a satisfactory test. It will be observed that the largest amount of ammonia was produced in the medium containing 2% of aminoid from egg albumin and the next largest amount from beef aminoid. Even with these substances the amount produced was less than with 4% bacto-peptone.

TABLE 5  
AMMONIA PRODUCTION FROM AMINOIDS

Source of Aminoid	Culture 16 H-1			Culture F 2-7		
	Percentage Aminoid in Infusion Broth			Percentage Aminoid in Infusion Broth		
	0.5	1.0	2.0	0.5	1.0	2.0
	Mg.	Mg.	Mg.	Mg.	Mg.	Mg.
Egg albumin.....	4.00*	7.56	16.25	0.0	0.84	0.98
Beef.....	4.06	6.02	11.35	0.28	0.14	0.56
Casein.....	2.94	1.12	0.56	0.14	0.0	0.56
Fibrin.....	1.96	2.10	2.38	0.28	0.28	0.14
Gelatin.....	2.66	0.70	2.10	0.28	0.14	0.42

\* Milligrams per 100 cc of medium; excess over control.

The aminoids represent materials so completely hydrolyzed that they do not give the Biuret reaction and contain amino acids which are probably in complex combinations. If it can be assumed that ammonia can be split off from amino acids which are in complex combinations, then from a survey of the amino acids which have been isolated from the various substances from which aminoids have been made it might be possible to throw some light on the ammonia-yielding substance. Before discussing this possibility a word relative to the amino-nitrogen content of the aminoid and bacto-peptone mediums will not be out of place.

Infusion broth was the basis of the mediums to which 4% bacto-peptone and various aminoids in different amounts were added. The reaction was adjusted to  $P_H$  7.5. The amino-nitrogen content of the various mediums containing different percentages of aminoids is shown in table 6 in terms of milligrams per 100 cc of medium.

It will be remembered that the ammonia-producing streptococcus culture, 16 H-1, formed about 20 mg. of ammonia-nitrogen in the infusion—4% bacto-peptone medium—and that the largest amount found in any of the aminoid mediums was 16.25 mg. with 2% aminoid

made from egg albumin. Reference to the amino-nitrogen content as listed in the foregoing shows that it was much higher in the 2% aminoid mediums than in the peptone medium. Even in the mediums with 1% aminoid the amino-nitrogen content was nearly as high as the amount in the peptone medium, and yet the ammonia was decidedly lower. Attention is called to these facts before further discussion of ammonia production from aminoids, because they show that the amino-nitrogen content of the aminoid and peptone mediums compared favorably.

TABLE 6  
AMINO-NITROGEN CONTENT OF VARIOUS MEDIUMS \*

Media	Percentage Aminoid		
	0.5	1.0	2.0
	Mg. per 100 C e	Mg. per 100 C e	Mg. per 100 C e
Infusion broth, egg albumin, aminoid.....	43.95	72.65	119.69
Infusion broth, beef, aminoid.....	44.51	71.39	119.67
Infusion broth, casein, aminoid.....	42.55	64.11	108.20
Infusion broth, fibrin, aminoid.....	43.67	70.83	118.56
Infusion broth, gelatin, aminoid.....	47.17	75.59	126.83
Infusion broth, no aminoid †.....	.....	.....	89.58

\* The amino-nitrogen content was determined by formol titration.

† Infusion broth, 4% bacto-peptone,  $P_H$  7.5.

TABLE 7  
APPROXIMATE AMOUNT OF AMINO-ACIDS FOUND IN VARIOUS MATERIALS

Amino Acids	Approximate Percentage Isolated from				
	Egg Albumin	Beef	Casein	Fibrin	Gelatin
Glycocoll.....	0.0	2.06	0.0	3.0	16.5
Alanine.....	2.2	3.7	1.5	3.6	0.8
Valine.....	2.5	0.81	7.2	1.0	1.0
Leucine.....	10.7	11.6	9.3	15.0	2.1
Aspartic acid.....	2.2	4.5	1.3	2.0	0.5
Glutamic acid.....	9.1	15.5	15.5	10.4	1.8
Cystine.....	0.3	...	0.1	1.1	0.0
Phenylalanine.....	5.2	3.7	3.2	2.5	0.4
Tyrosine.....	1.7	2.2	4.5	3.5	0.0
Proline.....	3.5	5.8	6.7	3.6	5.2
Tryptophane.....	...	Present	1.5	...	0.0
Histidine.....	1.7	2.6	2.5	...	0.4
Arginine.....	4.9	7.4	3.8	3.0	7.6
Lysine.....	3.7	7.5	5.9	4.0	2.7
Ammonia formed in culture medium containing 2% of aminoid.....	Egg Aminoid	Beef Aminoid	Casein Aminoid	Fibrin Aminoid	Gelatin Aminoid
	Yes	Yes	Trace	Very little	Very little

In table 7 is shown the approximate percentage of amino acids which have been isolated by various investigators from the material indicated, as is shown in the literature. In the lower part of the table is indicated the ammonia formation in aminoid mediums, based on our

results. A study of the table reveals the fact that ammonia formation in the different aminoid mediums considered in relation to the probable amino acids present gives no information as to the probable ammonia-yielding ingredients.

Further experiments were made with pure amino acids and acid amids which were added in slightly varying amounts to a 1% bacto-peptone, infusion-broth medium and inoculated with culture 16 H-1. An examination of table 8 shows that the addition of these substances did not appreciably affect ammonia production. Other substances such as sodium nucleate and yeast extracts proved to be of no value as a source of ammonia.

TABLE 8  
EFFECT OF ADDITION TO THE MEDIUM OF AMINO ACIDS AND ACID AMIDS ON AMMONIA PRODUCTION

Amino Acid or Acid Amid	Amount Added, Percentage	NH <sub>3</sub> -N Mg. per 100 C c; Excess over Control Mg.
Alanine.....	0.20	4.76
Aspartic acid.....	0.20	7.14
Cystine.....	0.20	6.86
Glycocoll.....	0.20	5.32
Histidine.....	0.30	6.16
Leucine.....	0.25	5.88
Phenylalanine.....	0.40	5.32
Tyrosine.....	0.40	5.60
Acetamide.....	0.20	5.60
Formamide.....	5 drops to 100 c c	5.18
1% bacto-peptone beef infusion.....		5.74

As has been stated before, there seemed to be a close correlation between the formation of ammonia and carbon dioxide from peptone. The amount of both increased with the increase in the peptone content of the medium, and different peptones which yield different amounts of ammonia gave correspondingly different amounts of carbon dioxide. If CO<sub>2</sub> results from a decarboxylation of an amino acid, we should expect to find amines present, but several analyses were made of different cultures and the presence of such bodies could not be demonstrated.

Our results seem to point to the probability that ammonia and carbon dioxide are derived in the degradation of some substance present in small amounts in some kinds of peptone. This is possibly accomplished through a process of deamidization, resulting in ammonia formation and a simultaneous or secondary oxidation resulting in carbon dioxide formation.

The possibility of the formation of CO<sub>2</sub> from the carbohydrate fraction of the protein molecule cannot be overlooked. This fraction,

although denied by some, is indicated by the Molisch reaction. However, the fact that the production of  $\text{NH}_3$  and  $\text{CO}_2$  are so closely correlated leads to the belief that the previous explanation is more probable.

Although it is to be regretted that ammonia and carbon dioxide production cannot, at present, be linked up with some definite and controllable ingredient of peptone, we believe that the formation of these substances, even though small in amount, represent fundamental differences in the physiology of the streptococci.

#### THE PRODUCTION OF CARBON DIOXIDE FROM DEXTROSE

Carbon dioxide produced by streptococci from peptone must not be confused with carbon dioxide from sugar. The gas-forming streptococcus isolated by Freudenreich<sup>10</sup> and named *Streptococcus kefir* by Migula,<sup>14</sup> has been shown by Evans<sup>8</sup> to be common in cheese. She did not, however, determine the source of the carbon dioxide. It has been shown by Sherman<sup>15</sup> that *Streptococcus kefir* produces carbon dioxide from lactose. He also showed that it was necessary to buffer the medium quite heavily to obtain the maximum carbon-dioxide production.

TABLE 9  
CARBON DIOXIDE PRODUCTION FROM DEXTROSE

Culture	No Dextrose Added	Percentage of Dextrose			
		0.5	1.0	1.5	2.0
X-4.....	C c	C c	C c	C c	C c
16 H-1.....	0.8*	9.5	14.5	16.5	18.8
	2.0	1.7	1.0	1.0	1.3

\* From 15 cc of medium.

Preliminary experiments with various cultures of streptococci showed that we had, in our collection of cultures, numerous organisms isolated from sour milk, which produced carbon dioxide from sugar. Neither  $\text{CO}_2$  nor  $\text{NH}_3$  was produced by these cultures from 4% bacto-peptone.

One culture known as X-4 was selected for a series of tests to show the difference in carbon-dioxide production from dextrose and from peptone. Eldredge fermentation tubes were filled with 15 cc of the following medium: infusion broth with 1% bacto-peptone, 1%  $\text{K}_2\text{HPO}_4$  and the reaction adjusted to  $\text{pH}$  7.5. This medium was used without sugar and also with various amounts of dextrose, as may be seen in table 9.

<sup>14</sup> System der Bakterien, Ed. 2.

<sup>15</sup> Jour. Bacteriol., 1921, 6, p. 127.

One set of tubes containing the medium without sugar and also with 0.5, 1.0, 1.5 and 2% of dextrose was inoculated with culture X-4 and another set with culture 16 H-1. These were incubated for 7 days at 30 C. For the sake of clearness we repeat that X-4 did not produce carbon dioxide or ammonia from 4% bacto-peptone infusion broth while 16 H-1 did produce them.

The results show clearly the difference between the two cultures. Culture X-4 showed only a trace of CO<sub>2</sub> in the medium without added dextrose, while the amount increased up to 18.8 c c with 2% dextrose. On the other hand, culture 16 H-1 showed a little more carbon dioxide in the medium without dextrose than in the medium with it. In this medium the amount of CO<sub>2</sub> formed from peptone was small because only 1% was used. Infusion broth with 4% bacto-peptone with 16 H-1 usually showed about 5 c c of CO<sub>2</sub> from 15 c c of medium, which is

TABLE 10  
COMPARISON OF GAS FORMATION IN ELDRIDGE AND SMITH FERMENTATION TUBES

Culture	Days of Incubation at 30 C.					
	1	2	3	4	6	7
CO <sub>2</sub> Production in Eldredge Tubes	C c	C c	C c	C c	C c	C c
Streptococcus X-4.....	10.5	14.5	16.0	15.6	16.1	16.1
B. coli.....	8.4	16.9	16.8	18.8	19.4	19.5
Gas in Smith Fermentation Tubes						
Streptococcus X-4.....	0	0	0	0	0	0
B. coli.....	+	+	+ 60% of arm	+	+	+ 60% of arm

the same amount as was used in this experiment. It is obvious from these results that it is a simple matter to determine whether a culture is a carbon-dioxide producer, and if so, to determine further whether it comes from peptone or dextrose. To do this it would only be necessary to determine CO<sub>2</sub> production in Eldredge tubes, one containing a medium consisting of infusion broth with 4% bacto-peptone and the other containing infusion broth with 1% bacto-peptone, 1% K<sub>2</sub>HPO<sub>4</sub> and 1% of dextrose, both mediums being adjusted to P<sub>H</sub> 7.5. A streptococcus which does not produce CO<sub>2</sub> will, of course, show none from either medium. One able to form it from peptone should show from 4 to 6 c c in the 4% bacto-peptone medium and much less from the sugar medium on account of the decreased peptone content. A streptococcus which can produce gas from dextrose should show quite large amounts in the dextrose medium but none or only a small amount in the tube with the 4% bacto-peptone medium.

It is interesting to observe that the streptococci are described as causing fermentations in carbohydrates without gas production. It is not surprising that carbon-dioxide production from peptone has been overlooked because of the peculiar conditions under which small but quite constant amounts are formed. There must be, however, some definite reason why gas formation from carbohydrates has not been more frequently observed since streptococci are quite common in sour milk and cheese which, under suitable conditions, produce as much carbon dioxide as the colon bacillus.

It is felt that the figures in table 10 give the reason for this oversight. The medium used in these experiments consisted of infusion broth with 1% bacto-peptone, 1% dextrose, 1% potassium dibasic phosphate ( $K_2HPO_4$ ) with a reaction of  $P_H$  7.5. Two sets of 6 Eldredge fermentation tubes were prepared and one set inoculated with a gas-forming streptococcus culture X-4 and the other set of 6 tubes with *B. coli*. At the same time two sets of Smith tubes containing the same medium were inoculated from the same two cultures. The carbon dioxide produced in the Eldredge tubes was determined by titration after 1, 2, 3, 4, 6 and 7 days' incubation at 30 C. As will be noted from table 10, the rate of carbon-dioxide production and the amount formed by the streptococcus and by *B. coli* were practically identical. In the lower part of the table is shown the gas production in the same medium and by the same cultures in Smith tubes. No gas was observed with the streptococcus, while the colon bacillus showed gas after 24 hours. When measured on the third and seventh day of incubation, the gas amounted to 60% of the closed arm. From these results it is evident that bacteria can produce as much  $CO_2$  as the colon bacillus without showing any gas in Smith tubes. This evidently explains why gas production by the streptococci from carbohydrates has not been more commonly found. No gas was observed in Smith tubes with the same medium and 2% of dextrose but with 5% dextrose the streptococcus culture X-4 showed 18% of gas in a Smith tube in 72 hours, and 25% after 7 days. On repeating this experiment no gas production was observed with X-4 in mediums containing 1, 2, 3, 4 and 5% of dextrose.

It is plain that there may be conditions under which gas production from carbohydrates by streptococci can be observed in Smith tubes. These conditions would, however, be unusual in that the medium would probably have to have a high sugar content and be heavily buffered. The defects of the Smith tube as a means of measuring  $CO_2$  have

been pointed out by numerous investigators. The chief objections to the tube have been summarized by Rogers, Clark and Davis<sup>16</sup> who point out that it is the  $\text{CO}_2$  which is measured with least accuracy by the Smith tube. It further appears that the tube is defective not only because of inaccuracies, but also because the production of large amounts of  $\text{CO}_2$  may not be detected by its use. Our results with the fermentation tube is the same as that of Fred, Peterson and Davenport,<sup>17</sup> who, while working with pentose fermenters, found that the fermentation tube was of no value for detecting gas formation as no gas was noted in the tubes, while it was found by other methods that  $\text{CO}_2$  was produced in large amounts.

No careful analysis was made of the gas from the streptococcus X-4, but the culture resembles *Streptococcus kefir* which, as Evans found, produced  $\text{CO}_2$  and no hydrogen. Since the colon bacillus produces both carbon dioxide and hydrogen and both these gases are found in the closed arm of the Smith tube, it is particularly interesting that the streptococcus, which as far as we know produces only  $\text{CO}_2$ , does not show evidence of this gas in the Smith tube, yet the same amount is formed by both this culture and *B. coli*. It is evident that the presence of hydrogen is an important factor in the appearance of  $\text{CO}_2$  in the closed arm of the Smith tube when both the gases are formed.

#### CARBON DIOXIDE PRODUCTION WITHOUT AMMONIA FORMATION

It has been shown that certain cultures of streptococci produce carbon dioxide and ammonia from peptone; that others do not produce either of these, and that still other cultures form  $\text{CO}_2$  from dextrose.

We wish now to call attention to certain types of streptococci which form carbon dioxide without ammonia. These cultures should not be confused with those which form  $\text{CO}_2$  from dextrose without  $\text{NH}_3$  production, as previously described.

The cultures listed in table 11, with the exception of the last two cultures (F 1-4 and F 1-6), were isolated from the udder of cows, but do not represent the typical udder streptococcus. The "F" cultures were isolated from cow feces and will be discussed later. Medium "A" consisted of infusion broth with 4% bacto-peptone, the reaction adjusted to  $\text{P}_\text{H}$  7.5. Medium "B" consisted of 4% bacto-peptone in distilled water with 0.5%  $\text{K}_2\text{HPO}_4$  and 0.2% dextrose. The initial reaction was  $\text{P}_\text{H}$  7.5.

<sup>16</sup> Jour. Infect. Dis., 1914, 14, p. 411.

<sup>17</sup> Jour. Biol. Chem., 1920, 42, p. 175.



It will be seen from the table that the udder cultures produced  $\text{CO}_2$  mostly in small amounts but no  $\text{NH}_3$  in either medium. We say "no ammonia" because the amounts found in excess of the control were so small as to be negligible. While the amount of  $\text{CO}_2$  formed by these cultures agreed in most cases with the amount formed by the type first described in this paper, it seems apparent that the source was not the same. It will be remembered that culture 16 H-1 produced  $\text{CO}_2$  and  $\text{NH}_3$ , both of which increased in amount with an increase in the content of bacto-peptone. In that case the  $\text{CO}_2$  and  $\text{NH}_3$  were evidently derived from some small fraction of the peptone.

TABLE 11

CARBON-DIOXIDE AND AMMONIA PRODUCTION BY CULTURES WHICH APPARENTLY LIBERATE CARBON DIOXIDE FROM PEPTONE OR DEXTROSE

Source	Culture Number	$\text{CO}_2$ : C c from 15 C c of Medium; Excess over Control	$\text{NH}_3\text{-N}$ : Mg. per 100 C c; Excess over Control	
		Medium A C c	Medium A Mg.	Medium B Mg.
Udder of cow	95 G	5.8	0.42	0.56
	101 G-2	2.6	0.28	0.28
	121 G-1	9.9	0.00	0.42
	121 G-2	3.9	0.14	1.69
	122 G-3	2.5	0.70	0.00
	132 G-1	3.1	0.00	1.40
	132 G-2	3.0	0.14	0.42
	134 G-1	3.1	0.42	0.00
	134 G-2	3.0	0.00	0.00
	135 G-1	2.7	0.00	1.27
	142 G-1	3.1	0.00	0.14
	144 G-1	4.5	0.28	0.28
	146 G-1	3.1	0.42	0.00
	148 G-1	2.7	0.28	0.42
	150 G-1	3.8	0.14	0.00
	68-2	4.0	0.42	0.00
Cow feces	F 1-4	3.0	24.66	0.00
	F 1-6	9.4	24.94	0.14

The source of  $\text{CO}_2$  produced by the streptococci now under discussion could not be definitely traced to peptone as the amount of  $\text{CO}_2$  was not correlated with the amount or make of peptone used in the medium, as was the case with culture 16 H-1. No evidence has been obtained to show that the  $\text{CO}_2$  was derived from dextrose, as was the case with many streptococci of which X-4 was a representative.

It must be admitted that no definite source of the  $\text{CO}_2$  produced by the cultures listed in table 2 has been found. The organisms were peculiar in many respects and grew with considerable difficulty in all mediums. We can only say, after an extensive study of this particular

problem which involved the use of many special mediums, that the results point to the salts of the organic acids as the possible source of the carbon dioxide.

Before closing this discussion attention must be called to two cultures of streptococci isolated from cow feces. These cultures F 1-4 and F 1-6 produced  $\text{CO}_2$  and  $\text{NH}_3$  in medium "A" but did not form ammonia in medium B. In medium A, however, F 1-4 and F 1-6 produced 3.0 and 9.4 c c of  $\text{CO}_2$ , respectively, from 15 c c of medium.

We feel that the difference in ammonia formation is important because, as will be shown later, these were the only two cultures of a collection of 485 streptococci which showed any distinct difference in ammonia formation in the two mediums. This difference in ammonia production is of further interest because the fecal cultures F 1-4 and F 1-6 were identical in most all other respects with the udder cultures 121 G-1, 132 G-1, 142 G-1, 144 G-1 and 146 G-1. These fecal cultures were slightly different in their  $\text{CO}_2$  producing ability from the udder cultures, but these differences were not sufficiently marked for discussion at this time.

#### DIVISION OF THE STREPTOCOCCI BY MEANS OF THE CARBON DIOXIDE AND AMMONIA TESTS

Before showing how various cultures of streptococci may be divided it may be well to briefly outline the various combinations of positive and negative carbon dioxide and ammonia tests as they have been found in our studies.

They may be listed in these combinations: A—Carbon dioxide and ammonia formation from bacto-peptone. B—No carbon dioxide and ammonia formation from bacto-peptone. C—Carbon-dioxide production from dextrose, no ammonia. D—Carbon-dioxide production not directly from dextrose, no ammonia. (In this case our results indicate that the  $\text{CO}_2$  was produced from the salts of organic acids; it may therefore come indirectly from sugar.) E—Carbon-dioxide production, ammonia formed in one medium but not in another.

Ammonia and carbon-dioxide formation by 485 cultures of streptococci were determined, but before taking up the results of this work the mediums and methods used should be mentioned. For the ammonia tests these two mediums were used:

Medium A  
Infusion broth  
4% bacto-peptone  
Reaction  $\text{P}_{\text{H}}$  7.5

Medium B  
4.0% bacto-peptone in distilled water  
0.2% dextrose  
0.5%  $\text{K}_2\text{HPO}_4$   
Reaction  $\text{P}_{\text{H}}$  7.5

Medium B was first used in our work, and the cultures incubated for 7 days at 37 C. Ammonia was determined by Folin's method, and the entire set of cultures was tested for ammonia production in this way. All of the cultures were also tested in medium A, and the ammonia in about 30 cultures was determined by Folin's method. With the remaining cultures ammonia was determined colorimetrically.

The ammonia was determined by the colorimetric method proposed by Thomas<sup>18</sup> as follows:

Reagents: A 4% solution of phenol in water.

A solution of sodium hypochlorite containing about 1% of available chlorine.

On mixing a dilute watery solution of an ammonia compound with the phenol and hypochlorite reagents, a blue color is gradually developed and reaches its maximum in about half an hour. The depth of color now remains almost constant for a long time and deepens slowly. Amino acids also give color reactions with these reagents, but only

TABLE 12  
COMPARISON OF AMMONIA DETERMINATIONS BY FOLIN'S AND COLORIMETRIC METHOD

Culture	Folin's Method	Colorimetric Method	
	NH <sub>3</sub> -N per 100 C c; Excess over Control. Mg.	Color after ½ Hour *	
		Eye, Mg.	Comparator Block, Mg.
F 1-4.....	6.86	3.0	5.0
F 1-4.....	6.86	3.0	5.0
F 9-1.....	23.82	21.0	22.5
16 H-1.....	24.24	20.0	25.0
93 G-1.....	23.68	20.0	22.5
95 G.....	7.84	5.0	7.6
95 G.....	8.27	5.0	7.6
8-5.....	23.68	20.0	22.5
X-2.....	26.06	21.0	26.0
X-2.....	25.64	21.0	26.0
X 52-2.....	21.58	18.0	22.5

\* All solutions were turbid.

in rather concentrated solutions. Glycocoll in a 1:10,000 solution gives only pale green, whereas ammonia salts react in a dilution of 1:500,000. The peptone medium used in our experiments does not contain sufficient free amino acids to interfere with the ammonia reaction in the dilution used for making the test.

Test: Two-tenths cc of the medium are diluted to 8 cc with water to which are added 1 cc of phenol reagent and 1 cc of hypochlorite reagent; these are mixed well and allowed to stand for 30 minutes. The resulting coloration is green instead of blue, owing to the yellow color of the medium.

<sup>18</sup> Bull. Soc. Chim., Series 4, 1912. 11, p. 726.

Table 12 shows the results obtained by Folin's method and the phenol-hypochlorite reagent. A solution of ammonium sulphate was prepared by dissolving 0.9433 gm. of pure salt in 1 liter of ammonia-free water and diluting 100 c c of this to 1 liter. One c c of the latter solution contains 0.02 mg. of nitrogen. Various amounts of this solution were added to 0.2 c c of the control medium in preparing the standard solutions, the volume in each tube being brought up to 8 c c for comparison by the eye. With the comparator block no control medium was added to the standard. Column 1 gives the milligrams of ammonia nitrogen in excess over the control found by Folin's method; column 2 shows the results obtained by looking through the column of liquid in the tube; while column 3 gives the results found in using the comparator block. It will be seen that the results obtained by looking through the liquid are low owing to the turbidity of the solution, while the controls of course are clear. With the comparator block, results are fairly accurate because a turbid solution of the medium is placed in the block. The advantage of the method lies in its rapidity when a large number of cultures are to be examined for the excess of ammonia nitrogen produced by the bacteria. Furthermore, the test can be used in the presence of proteins which interfere with Nessler's reagent.

Carbon-dioxide production by all the cultures was determined in medium A described previously, but the cultures which showed no ammonia were tested for  $\text{CO}_2$  formation in the following medium.

Medium C  
4.0% bacto-peptone in distilled water  
0.5% dextrose  
0.5%  $\text{K}_2\text{HPO}_4$   
Reaction  $\text{P}_{\text{H}}$  7.5

Many cultures which did not form  $\text{CO}_2$  in medium A produced it from the dextrose in this medium. The cultures which produced  $\text{CO}_2$  from 4.0 bacto-peptone of course formed it in the medium C with dextrose, but the amount was less.

When ammonia determinations were made by Folin's method, amino-nitrogen determinations were also made by means of formol titration, which showed some interesting facts.

Hydrogen-ion determinations, by colorimetric means, were made of each culture, but the results are not included in this paper since they proved to be of no particular significance.

The results of the application of the  $\text{CO}_2$  and  $\text{NH}_3$  tests to a large number of cultures of streptococci are shown in table 13. It will be observed that 3 different combinations of the tests were found in a study of 124 cultures isolated from sour milk from individual farms. The samples of milk from which the cultures were obtained were mixed samples from the milk from one farm. Each sample was allowed to stand at room temperature until it had developed an acidity of from 0.3 to 0.5%. The sample was then plated on dextrose-extract peptone agar and cultures of streptococci isolated. The cultures were isolated from samples of milk from 50 different farms. Seventy-seven of the total 124 cultures produced  $\text{CO}_2$  from dextrose and formed no  $\text{NH}_3$ . Thirty-four cultures formed  $\text{CO}_2$  from peptone, none from dextrose and also formed  $\text{NH}_3$ . Thirteen cultures produced neither  $\text{CO}_2$  nor  $\text{NH}_3$ .

It is interesting to note how the results changed when streptococci isolated from mixed samples of sour milk from many farms were examined. These cultures came from about 25 mixed samples of milk, each sample representing a mixture from many farms. These samples were allowed to stand at room temperature until the acidity had increased to about 0.6%. These mixed samples were from the same farms from which the previous set of cultures were isolated. Of the 96 cultures isolated only 6 formed  $\text{CO}_2$  from dextrose with no  $\text{NH}_3$ , while 90 produced  $\text{CO}_2$  from peptone and formed  $\text{NH}_3$ . From this it seems evident that the proportion of the types of streptococci found in sour milk from individual farms varies from that of mixed samples from many farms. It is possible, however, that the difference was due to the fact that in the latter case the milk was allowed to reach a slightly higher acidity. Three combinations of the carbon dioxide and ammonia tests A, B and C, were observed among the cultures isolated from sour milk.

The 78 cultures of streptococci from cow feces showed 3 combinations, A, B, and E. No cultures were found which gave the C combination, while 75 of the 78 gave the B type, that is, no  $\text{CO}_2$  and no  $\text{NH}_3$ . Two cultures were found which varied from any others in our collection in that they produced  $\text{CO}_2$  and  $\text{NH}_3$  in one medium but not in another.

The majority of the streptococci from the udder of cows showed the A combination, but 20 gave the D type. It will be remembered that these cultures produced  $\text{CO}_2$  from the peptone medium A but formed no  $\text{NH}_3$  in either medium A or B. The  $\text{CO}_2$  apparently did not come

from the peptone or dextrose, but may have come from the salts of organic acids. These streptococci were found only in the udder.

The hemolytic streptococci from pathologic sources and normal human throats gave the same combination as the majority of those from the udder but were different in other reactions. Among the cultures isolated from commercial starters it was found that 4 produced  $\text{CO}_2$  and  $\text{NH}_3$  from the peptone, while the other 4 showed neither.

Throughout our work the excess of amino nitrogen over the control medium was determined with all cultures, and the uniformity of the difference between different collections of the same type of organism was striking. Medium B was used because it showed the greatest difference between cultures. The excess of amino nitrogen over the control was averaged and is shown in table 13.

TABLE 13  
STREPTOCOCCI FROM VARIOUS SOURCES GROUPED BY THE CARBON-DIOXIDE AND  
AMMONIA TESTS

Source of Cultures	Number of Cultures	CO <sub>2</sub> Production		NH <sub>3</sub> Production		Average Amino-N Mg. per 100 C c; Excess over Control; Medium B Mg.	Combina- tion Letter
		Pep- tone Medium A	Dex- trose Medium C	Pep- tone Dex- trose Medium B	Infu- sion Pep- tone Medium A		
Sour milk, individual farms	77	—	+	—	—	1.56	C
	34	+	—	+	+	12.81	A
	13	—	—	—	—	11.50	B
Sour milk, mixed sam- ples from many farms	6	—	+	—	—	2.15	C
	90	+	—	+	+	13.02	A
Cow feces	75	—	—	—	—	14.20	B
	1	+	—	+	+	19.20	A
	2	+	—	—	+	3.01	E
Udder of cow	95	+	—	+	+	14.51	A
	20	+	—	—	—	2.97	D
Hemolytic, from patho- logic sources	46	+	—	+	+	—	A
Hemolytic, from normal human throats	18	+	—	+	+	—	A
Active lactic streptococci from commercial starters	4	+	—	+	+	14.09	A
	4	—	—	—	—	4.99	B
Total	485						

Cultures which gave the C combination showed little amino nitrogen regardless of source. Those which gave the A combination showed a greater excess of amino nitrogen, which in most cases was similar to the amount formed with cultures giving the B combination.

TABLE 14

COMPLETE RESULTS OF CARBON DIOXIDE, AMMONIA AND AMINO-NITROGEN PRODUCTION BY STREPTOCOCCI AS GROUPED IN TABLE 13

Source of Cultures	Number of Cultures	Production of CO <sub>2</sub>				NH <sub>3</sub> -N per 100 C c; Excess over Control				Mg. Aminonitrogen; Excess over Control		Combination Letter
		Infusion Peptone Medium A		Dextrose Peptone Medium C		Infusion Peptone Medium A		Peptone Dextrose Medium B		Peptone Dextrose Medium B		
		Average, C c	Range, C c	Average, C c	Range, C c	Average, Mg.	Range, Mg.	Average, Mg.	Range, Mg.	Average, Mg.	Range, Mg.	
Sour milk, individual farms	77	0.8	0.0-1.1	6.7	3.9-7.8	0.01	0.01	0.06	0.0 - 0.42	1.56	0.0 - 3.92	C
	34	4.9	2.9-6.2	—*	.....	22.04	20.0-24.0†	21.33	18.00-27.74	12.81	7.56-20.65	A
	13	0.5	0.0-1.5	0.1	0.0-1.0	0.01	0.01	0.12	0.0 - 0.70	11.51	6.58-15.26	B
Sour milk, mixed samples from many farms	6	0.6	0.3-0.9	7.2	6.2-9.9	0.01	0.01	0.30	0.0 - 0.56	2.15	0.0 - 8.40	C
	90	4.4	2.6-7.3	—*	—	21.81	20.0-24.0†	20.12	7.57-22.42	13.02	2.23-18.74	A
Cow feces	75	0.3	0.0-0.5	0.3	0.0-0.6	0.01	0.01	0.12	0.0 - 0.56	14.20	12.46-16.38	B
	1	3.9	—	0.3	0.2-0.4	23.82	21.10-24.94	20.74	—	—	—	A
Udder of cow	2	8.0	7.4-8.5	0.3	—	24.52	24.10-24.94	1.19	1.12-1.26	3.01	2.38-3.64	E
	95	4.6	2.8-6.8	—*	—	21.31	20.0 - 22.0†	22.39	12.80-24.94	14.51	8.38-24.76	A
Hemolytic, from human pathologic sources	20	3.8	2.0-9.9	—*	—	0.17	0.14 - 0.42	0.51	0.0 - 1.97	2.97	0.0 - 8.12	D
	36†	4.0	3.1-6.2	—*	—	16.54	13.0 - 18.0†	—	—	—	—	A
Hemolytic, from normal human throats	10†	5.7	4.2-7.4	—*	—	27.48	23.54-29.14	—	—	—	—	A
	18	4.2	3.5-5.9	—*	—	14.61	10.0 - 18.0†	—	—	—	—	A
Active lactic streptococci from commercial starters	4	4.8	4.2-5.2	—*	—	19.38	18.77-20.17	20.91	19.76-21.58	14.09	13.28-15.24	A
	4	0.6	0.5-0.7	0.7	0.5-1.2	0.42	0.14 - 0.84	0.47	0.0 - 1.42	1.49	3.29-6.58	B

\* Carbon dioxide in this medium not determined with all cultures.

† Ammonia estimated by colorimetric means.

‡ Represents two sets of cultures sent to us from different laboratories.

§ No determinations made.

It is felt that these differences in amino-nitrogen content of culture medium caused by the growth of streptococci is significant particularly since cultures, some of which produce ammonia and some of which do not, show about the same amino-nitrogen figures.

For those who wish to follow the results of the carbon dioxide and ammonia test and amino-nitrogen figures more carefully, we have condensed our results as shown in table 14. The average figure and range in results is given for carbon dioxide, ammonia and amino

TABLE 15

THE SEPARATION BY MEANS OF AMMONIA AND CARBON-DIOXIDE TESTS OF CULTURES GIVING SIMILAR REACTIONS ACCORDING TO THE METHODS COMMONLY USED FOR CLASSIFICATION

Culture No.	Source of Culture	Litmus Milk	Dextrose	Lactose	Saccharose	Salicin	Mannite	Raffinose	Inulin	CO <sub>2</sub>		NH <sub>3</sub> Medium A
										Dextrose, Medium C	Pep-tone, Medium A	
1	Sour milk	Slight acid	+	+	+	—	—	+	—	+	—	—
2	Sour milk	Slight acid	+	+	+	+	—	+	—	+	—	—
3	Cow feces	Slight acid	+	+	+	+	—	+	—	—	—	—
4	Cow feces	Not coagulated but decolorized	+	+	+	—	—	+	—	—	+	+
5	Udder of cow	Acid coagulated	+	+	+	—	—	+	—	—	+	—
6	Sour milk	Acid coagulated ½ decolorized *	+	+	+	+	+	—	—	—	+	+
7	Sour milk	Acid coagulated decolorized *	+	+	+	+	+	—	—	—	—	—
8	Sour milk	Acid coagulated decolorized *	+	+	—	—	—	—	—	—	+	+
9	Sour milk	Acid coagulated decolorized *	+	+	—	—	—	—	—	—	—	—
10	Sour milk	Acid coagulated decolorized *	+	+	+	+	+	+	—	—	—	—
11	Udder of cow	Acid coagulated decolorized *	+	+	+	+	+	+	—	—	+	—

\* Litmus decolorized, milk reaction after 7 days.

nitrogen. No discussion of the results is necessary further than to say that the averages and ranges show the uniformity in results obtained by cultures giving the different tests.

#### APPLICATION OF THE CARBON-DIOXIDE AND AMMONIA TESTS TO THE STREPTOCOCCI

The manner in which cultures from various sources were divided by these tests has been shown, and there now remains for discussion some more specific applications.



Attention is called to table 15 which contains a list of cultures with their litmus milk reactions and their fermentation, carbon dioxide and ammonia tests. Cultures 1 and 2 from sour milk showed the same reactions in litmus milk as culture 3 from cow feces. In fermentation tests, culture 1 varied from culture 3 only in the fermentation of salicin. From these physiologic tests, which are most commonly employed for differentiating streptococci, it would probably be considered that these cultures were identical. The tests at the right of table 15, however, tell another story.

The streptococci from sour milk, cultures 1 and 2, produced  $\text{CO}_2$  from dextrose and formed no  $\text{NH}_3$ , while culture 3, from cow feces, did not form either carbon dioxide or ammonia. Culture 1 was representative of a majority group isolated from sour milk from individual farms while the fecal streptococcus, culture 3, represented the typical streptococcus of cow feces. From the results of ordinary tests these organisms of the culture 1 type found in sour milk would probably be considered fecal streptococci but as the carbon dioxide and ammonia tests show, they were entirely different.

Cultures 4 and 5 from cow feces and the udder of the cow differed somewhat in their litmus-milk reactions, and agreed in fermentation tests, yet were quite different from the first three cultures in their  $\text{CO}_2$  and  $\text{NH}_3$  test.

Of the other streptococci isolated from sour milk, cultures 6 and 7 were identical by the ordinary tests but quite different in their  $\text{CO}_2$  and  $\text{NH}_3$  formation. The same may be said of cultures 8 and 9.

By the ordinary tests, culture 10, from sour milk, would be considered the same as culture 11 isolated from the udder of a cow, but the  $\text{CO}_2$  test shows they were quite different.

It is our opinion that the carbon-dioxide and ammonia tests will be extremely valuable in any study which involves the physiologic characteristics of the streptococci. Undoubtedly they will provide a means for separating cultures which are grouped together by the ordinary tests.

#### MEDIUMS AND METHODS FOR THE TESTS

So many mediums have been mentioned in this paper that it is believed it will be of advantage to the reader to suggest at this point the best means for making the tests.

For the ammonia test the following mediums are used:

Medium A	Medium B
Infusion broth	4.0% bacto-peptone in distilled water
4.0% bacto-peptone	0.2% dextrose
Reaction $P_{H}$ 7.5	0.5% $K_2HPO_4$
	Reaction $P_{H}$ 7.5

In our work the cultures have been incubated 7 days, but ammonia formation apparently reaches its maximum in 24 hours. The amount can be determined by Folin's method, but for routine work the colorimetric method previously described is suggested.

Usually when ammonia is formed in medium A it is also found present in medium B, but two cultures were found which did not produce ammonia in the latter medium. It would be desirable, therefore, to use both mediums. The incubation temperature can be either 30 C. or 37 C.

Carbon dioxide may be produced from dextrose or from peptone or possibly from organic acid salts, and for this reason the use of two different mediums is necessary.

CO <sub>2</sub> from peptone or organic acid salts	CO <sub>2</sub> from dextrose
Medium A	Medium S
Infusion broth	Infusion broth
4.0% bacto-peptone	1.0% bacto-peptone
Adjust reaction to $P_{H}$ 7.5	1.0% $K_2HPO_4$
	1.0% dextrose
	Adjust reaction to $P_{H}$ 7.5

It is suggested that 15 c c of these mediums be placed in Eldredge tubes; of course larger amounts can be used up to the capacity of the tubes. When using medium A, 10 c c of N/10  $Ba(OH)_2$  is sufficient to neutralize the  $CO_2$  produced. With medium S at least 15 c c of N/10  $Ba(OH)_2$  should be used if the amount of medium is 15 c c.

A streptococcus which forms  $CO_2$  from peptone in medium A should show from about 4 to 6 c c of  $CO_2$  and with medium S not much more than 1 c c. This is due to the decreased content of peptone when bacto-peptone is used. As has been shown previously, bacto-peptone should be used when ammonia production or carbon dioxide formation from peptone is being determined.

A streptococcus which forms  $CO_2$  in medium A but shows no  $NH_3$  formation is distinctly different from one which shows  $CO_2$  and  $NH_3$ .

In this case, as mentioned, our results indicate that the organic acid salts may be the source of the carbon dioxide, but this has not been definitely proved.

A streptococcus which forms  $\text{CO}_2$  from dextrose will show about 10 c c of  $\text{CO}_2$  from 15 c c of medium S, but not more than 1 c c from medium A. The Eldredge tubes should be incubated for at least 3 days either at 30 C. or 37 C.

#### SUMMARY AND CONCLUSIONS

The determination of ammonia can easily be made a routine procedure by means of a colorimetric method.

Carbon-dioxide production by the streptococci seems to have been largely overlooked because of the use of the fermentation tubes commonly used. The Eldredge fermentation tubes measure quite accurately small amounts of carbon dioxide which may be given off from cultures in liquid mediums. Their use is highly recommended.

In suitable mediums streptococci produced ammonia and carbon dioxide.

It has been shown that with many streptococci the production of ammonia and carbon dioxide is correlated. In such cases both are formed apparently from some ingredient of peptone present in small amounts. Not all makes of peptone can be used.

Certain streptococci formed carbon dioxide but no ammonia. Some experiments indicated that the source of this carbon dioxide may have been the organic acid salts.

Streptococci also formed carbon dioxide from dextrose. These did not form ammonia in the mediums used in this work.

The ammonia and carbon-dioxide tests divided the streptococci studied as follows:

- Some produced neither ammonia nor carbon dioxide from peptone.
- Some produced both ammonia and carbon dioxide from peptone.
- Some produced no ammonia but formed carbon dioxide, which did not come from peptone or dextrose.
- Some produced no ammonia but formed carbon dioxide from dextrose.

The amount of ammonia formed was small and the same may be said of the carbon dioxide formed from peptone. However, the amounts were quite uniform under definite conditions. It is believed

that the formation of these substances represents fundamental differences in the physiology of the streptococci, and that they are highly significant.

By the use of the ammonia and carbon-dioxide tests, it has been found to be possible to separate groups of streptococci which, on the basis of the ordinary physiologic tests, were considered identical.

## ANTIRABIC VACCINATION BY MEANS OF DESICCATED VIRUS

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Shackell's<sup>1</sup> simple method for preserving the infectivity of rabic virus, made the basis of further study by himself and Harris,<sup>2</sup> and finally elaborated by Harris<sup>3</sup> into a simple measure for the preparation of material for antirabic prophylactic vaccination, represents advances of great importance in the study of rabies. Those experienced with the old Pasteur dried cord method know quite well the financial outlay necessary and the amount of time and energy consumed in the preparation of material for treatment. Any measure that allows the production of a potent effective agent for antirabic vaccination in a short time, and the preservation of this agent over indefinite periods, is a signal accomplishment.

For a number of years these laboratories have given rabies prophylactic treatment with material prepared along the lines indicated by Harris. The results have been so gratifying that we desire to tabulate the cases treated for the past six years, and to add a few observations which have been made on various aspects of the desiccated virus. As slight modifications have been made in the technic originally proposed by Harris, it may be of value to describe in some detail the methods used in the production of virus.

### PREPARATION OF VIRUS

Full-grown, healthy rabbits, averaging 2,200 gm., are inoculated intracerebrally with an emulsion of desiccated fixed virus, the amount used being usually 0.004 mg. of material in 1 c c of sterile salt solution. Injection is made after trephining, the material being deposited by means of a 24 gage needle directly into the lateral ventricles. After a period of 6 to 7 days the animals develop typical symptoms, and when complete paresis has intervened, they are killed by ether narcosis. The cord and brain are removed under aseptic conditions and placed in

Received for publication May 9, 1921.

<sup>1</sup> Am. J. Phys., 1919, 24, p. 325.

<sup>2</sup> J. Infect. Dis., 1911, 8, p. 47.

<sup>3</sup> Ibid., 1912, 10, p. 369; 1913, 13, p. 155; Ann. l'Inst. Pasteur, 1912, 26, p. 372.

a petri dish 100 mm. in diameter, covered with cold sterile salt solution and the membranes then carefully stripped off by needles. The removal of the membranes is necessary, as this later allows division of the material into finer particles. The cord and brain are placed in a mortar and brought into a coarse paste by the addition of a little salt solution. Carbon dioxide snow prepared in the usual manner is then added with constant mixing and triturating until solidification of the mass has taken place. When this is accomplished the material is placed in an ordinary meat grinder, previously sterilized and kept at a temperature of about  $-12^{\circ}\text{C}$ . for a few hours, a small amount of  $\text{CO}_2$  again added and quick grinding accomplished. The ground material is spread in a thin layer over a cold sterile porcelain perforated plate which fits a 25 cm. pyrex glass Scheibler desiccator, previously sterilized and kept for a few hours at a temperature of  $-12^{\circ}\text{C}$ . A layer of phosphoric anhydride 2 cm. deep covers the bottom of the desiccator and acts as the dehydrating agent. A vacuum of 2 mm. of mercury is quickly produced, and the desiccator placed at a temperature of  $-12^{\circ}\text{C}$ . to  $-18^{\circ}\text{C}$ . Invariably, if the material has been spread in a thin layer (averaging about 4 mm. in thickness), complete desiccation is accomplished within 36 hours. After desiccation the material is scraped off of the porcelain plate by means of a sterile spatula, collected in a sterile beaker and pulverized in a moisture-free atmosphere by means of a sterile glass rod. The powdered material is placed in pyrex glass tubes 125 mm. long by 10 mm. in diameter. These tubes are quickly sealed by flame and stored in a dark place at a maximum temperature of  $-10^{\circ}\text{C}$ ., the usual average temperature being  $-12^{\circ}\text{C}$ . to  $-15^{\circ}\text{C}$ .

The question of sterility of material prepared in this manner must of course be considered. It is conceivable, notwithstanding the great amount of care exercised in handling the virus and the precautions taken in assuring that all apparatus used in its preparation is sterile, that at times contamination may take place. Extraneous organisms entering the material during its preparation are subjected to desiccation and low temperature, which prevent their multiplication but do not impair their viability, as Swift has admirably shown. The question of sporulating organisms deserves especial attention. It is possible, however improbable, for spores of such organisms as *B. tetani* to be present in the carbon dioxide used in the preparation of the snow. Although the injection of such spores in man in the great majority of cases would be followed by no ill effects, the impossibility of such a

happening is assured by the careful cultural control of every batch of virus prepared. Aerobic and anaerobic cultures are prepared from material obtained from each single animal used, and also from each single tube of the finally prepared desiccated virus. It is believed that these precautions have not been taken in vain, as to this date there have never been even local infections in patients after subcutaneous injections of virus.

#### STANDARDIZATION OF DESICCATED VIRUS

It has appeared necessary to establish certain arbitrary standards in dealing with desiccated rabies virus. So little being known of the agent concerned, the problem is one of great difficulty. The established methods of standardizing on the basis of protection or on the basis of neutralization of toxic substances are of course not available. Throughout his work Harris used as a standard basis a "unit" which represented the smallest amount of desiccated material necessary to infect a rabbit. We employ the same standard, retaining for it the term "minimal infective dose" and defining it as the least amount of desiccated material which within 5 days after preparation will cause paresis in a 2,400 gm. rabbit on the seventh day following intracerebral injection. It is not claimed that the standard is of any great degree of accuracy, as naturally a great variety of factors enter into the question of infection. However, it is believed to be an advantage to have some fixed unchanging basis on which to establish the degree of infectivity of any particular batch of virus. Every batch of virus prepared is standardized along these lines and there never has been any difficulty in producing a virus with a "minimal infective dose" of between 0.002 and 0.004 mg. when desiccation has been accomplished within 48 hours. If desiccation has taken longer the number of "minimal infective doses" will be greatly reduced per mg. of material.

#### RELATION OF TEMPERATURE AND AGE OF DESICCATED VIRUS TO INFECTIVITY

Desiccated virus may be kept in the dark at temperature of  $-12^{\circ}\text{C}$ . over long periods of time without any demonstrable loss of infectivity. Thus, one lot stored under the conditions indicated in the foregoing still contains the same number of "minimal infective doses" per mg. (500) as when prepared 700 days ago. At refrigerator temperature ( $8^{\circ}\text{C}$ . to  $12^{\circ}\text{C}$ .) infectivity may last as long as approximately 3 years.

At higher temperatures infectivity is rapidly lost, never lasting in our experience at room temperature (23 C. to 28 C.) longer than about 60 days.

#### RELATION OF INFECTIVITY OF DESICCATED VIRUS TO IMMUNIZING PROPERTIES

Harvey and McKendrick's <sup>4</sup> statement "that the immunizing power of a given portion of rabies cord is a function of the unkilld remnant of rabies virus which is contained in that cord" is open to criticism. Material which has lost all infectivity is capable of producing immunity. Babes' use of heat, Fermi's use of carbolic acid in preparing material for vaccination, and the results achieved with such material in prophylactic vaccination, all tend to disprove Harvey and McKendrick's contention. Our own experiences are in accord with Harris'. It has been possible to protect experimental animals with noninfective material. With one batch of desiccated virus, which had lost all infectivity after 200 days at a temperature ranging between 24 C. and 28 C., animals were protected regularly, but it was necessary to use large amounts of material over long periods of time. As infective virus protects in much smaller and fewer doses, and in view of its apparent harmlessness for human administration, its use is preferred.

#### METHODS OF ADMINISTRATION

Treatment of patients bitten by rabid animals is done in these laboratories by giving subcutaneously an initial dose of 250 "minimal infective doses," doubling the dose daily until a maximum of 2,000 "minimal infective doses" is reached. We never consider the ratio existing between infective and noninfective material in the virus which is used. As a rule, the virus is 2 or 3 months old before being used, but as it loses practically none of its infective powers during that time, we are always dealing with a freshly prepared virus as far as infectivity is concerned. Usually a total of 11 treatments of 17,750 "minimal infective doses" are given, except in severe penetrating injuries of the face and scalp when 15 treatments of a total of 25,750 "minimal infective doses" are given. We consider the smaller dose over a long period of time more efficacious in establishing immunity, although we have repeatedly protected animals by means of a few massive injec-

<sup>4</sup> Theory and Practice of Antirabic Vaccination, 1907.



tions of fixed desiccated virus. In children under 3 years half the number of "minimal infective doses" indicated as an adult treatment is given.

#### STATISTICAL STUDY OF TREATED CASES

During the last six years 1,538 patients have been treated in these laboratories with virus prepared as outlined. Tables 1 and 2 give the

TABLE 1  
STATISTICAL SURVEY OF CASES

	1915	1916	1917	1918	1919	1920	Total
Number of patients applying for treatment.....	859	938	1,040	993	879	667	5,376
Number of cases treated.....	223	148	324	369	300	174	1,538
Attacking animals showing positive rabies:							
Microscopic test.....	89	58	153	149	124	89	662
Biologic test.....	0	0	0	10	0	0	10
Attacking animals not located.....	34	90	171	210	176	85	866
Rabies developed after treatment....	0	0	0	0	1	0	1

TABLE 2  
DISTRIBUTION OF LESIONS IN PATIENTS INJURED BY ANIMALS PROVED TO BE RABID

	1915	1916	1917	1918	1919	1920	Total
Face.....	9	4	8	9	16	7	53
Scalp.....	1	0	3	2	3	1	10
Arm.....	22	14	21	20	16	8	101
Hand.....	18	16	76	85	43	38	276
Leg.....	35	21	39	35	36	21	187
Foot.....	4	3	6	8	10	4	35

TABLE 3  
DISTRIBUTION BY AGE OF TREATED PATIENTS

	1915	1916	1917	1918	1919	1920	Total
Under 1 year.....	0	0	1	1	1	0	3
1- 3 years.....	19	17	29	21	27	19	132
3- 5 years.....	16	9	32	29	25	17	128
5-10 years.....	66	54	82	88	75	36	401
10-15 years.....	37	21	46	48	45	34	231
15-20 years.....	14	12	23	35	17	14	115
20-30 years.....	25	14	42	48	35	21	185
30-40 years.....	21	10	30	39	35	13	148
40-50 years.....	14	9	21	31	21	15	111
50-60 years.....	9	2	15	21	16	4	67
60-70 years.....	2	0	3	8	3	1	17
Total.....	223	148	324	369	360	174	1,538

cases and classify sites of injury in patients injured by rabid animals. Table 3 classifies the treated cases according to age. A positive diagnosis of rabies was made in suspected animals only on the finding of Negri bodies ("microscopic test") or on the reproduction of lesions after the inoculation of test animals with brain emulsions ("biologic

test"). The biologic test we find of little value. In cases of rabies, Negri bodies are always found in such large numbers as to preclude the possibility of error. We have found in the case of badly infected and decomposed brains the biologic test to be practically useless, as invariably the animals succumb to purulent meningitis when injected with emulsions made of such material. A number of patients have been treated, without possibility of examination of the brain, due to impossibility of locating the offending animal or due to the destruction of the animal. In none of the latter cases has untoward results followed treatment. About 25% of the patients developed a malaise and slight elevation of temperature during the first few days of treatment, and approximately 10% of cases developed a disagreeable urticarial-like eruption, which we attribute to sensitization of foreign protein. We have not noted this phenomenon nearly as frequently as Geiger<sup>5</sup> using the old Pasteur dried cord method. No cases of paralysis have developed so far in our treated cases, an important fact and one at variance with the experience of observers using the old dried cord method. Mejia,<sup>6</sup> however, places the paralysis occurring at the Buenos Aires Pasteur Institute as low as one case in 2,142.

The one death from rabies after treatment recorded in our series occurred in a boy 7 years of age. He had been bitten extensively about the face, neck and hands by a dog which was proved rabid by microscopic examination of brain sections. The day following injury treatment was begun, and lasted over a period of 15 days, the patient receiving in all 25,750 "minimal infective doses." The seventeenth day after injury (the day following the termination of treatment) the child showed symptoms of rabies, and died on the third day. Although post mortem examination was not obtainable, the case was undoubtedly one of rabies.

#### CONCLUSIONS

The Harris method of preparing rabies virus with some modifications has been used for six years in these laboratories.

A virus containing 300 to 500 "minimal infective doses" (for 2,400 gm. rabbits) per milligram is readily produced.

When stored in the dark at a maximum temperature of—10 C. no loss whatever to the infectivity of desiccated virus can be demonstrated for periods over 2 years. At temperatures of 8 to 12 C. infectivity

<sup>5</sup> Jour. Am. Med. Assn., 1916, 67, p. 321.

<sup>6</sup> Semaine med., 1917, 24, p. 10.

lasts approximately 3 years. At temperatures of 23 C. to 28 C. infectivity lasts approximately 60 days.

Noninfective desiccated virus is not necessarily nonprotective. Protection, however, is more constant and more readily accomplished by the use of infective virus.

Adults are given 11 treatments of a total of 17,750 "minimal infective doses," except in severe penetrating injuries of the head when 15 treatments of a total of 25,750 "minimal infective doses" are administered.

The cases of 1,538 treated patients are reported, 697 injured by animals proved to be rabid. One death following complete treatment is reported.

No paralysis or other untoward effects have been noted in the series of patients treated.

The results reported, on the basis of comparison with similar reports on the use of the original Pasteur dried cord method, argue for the efficaciousness and safety of the desiccated virus method of prophylactic antirabic vaccination.

## FURTHER OBSERVATIONS ON "BLACKHEAD" IN TURKEYS

ERNEST EDWARD TYZZER; MARSHAL FABYAN,  
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The investigation of blackhead, continued during the summer of 1920, has yielded additional data which, although contributing to the general knowledge of the disease, leave certain practical questions still unanswered. Blackhead was transmitted by subcutaneous and intramuscular inoculation throughout the season, thus confirming previous results.<sup>1</sup> The resistance of the virus to variations of temperature, and the question of its discharge from the mucous membranes were studied. Attempts to produce the disease other than by the previously employed method of inoculation of acute blackhead lesions have yielded some facts concerning the characteristics and distribution of *Histomonas meleagridis*, the parasite of blackhead. A study was also made of the relative frequency in normal and infected turkeys of the parasitic round-worm *Heterakis papillosa*.

On account of the varied character of the experiments, the data obtained will be summarized under separate headings.

### EFFECT OF TEMPERATURE ON VIRUS

The resistance of the virus, as it occurs in the lesions of acute blackhead, was determined by inoculating turkeys with infectious material after its exposure to different temperatures. Livers showing lesions characteristic of acute blackhead were removed under aseptic precautions from freshly killed young turkeys and portions of the infected foci were distributed in sterile Petri dishes. Bits of these lesions after being subjected to freezing, refrigerator, or room temperature for definite periods of time, were then subcutaneously inoculated into turkeys from 3 to 6 weeks old. The results are given in table 1.

Received for publication May 16, 1921.

<sup>1</sup> Tyzzer, E. E., and Fabyan, M.: Jour. Infect. Dis., 1920, 27, p. 207.

It is obvious from these results that the active virus is present in the lesions of a freshly killed turkey with acute blackhead, and that it probably deteriorates more rapidly at room temperature than at lower temperature. At refrigerator temperature—5 C.—it may remain alive

TABLE 1  
EFFECT OF TEMPERATURE ON VIRUS

Blackhead Virus	Date	Treatment of Virus	No. of Turkey Inoculated	Age in Days	Weight in Grams	Result
Turkey 20.86..... (Spontaneous) Liver lesions	June 28	None. Used at once	4	21	260	Died of inoculated blackhead on 12th day
Ibid. ....	.....	48 hours at 5 C.	5	23	280	Died of inoculated blackhead on 13th day
Ibid. ....	.....	48 hours at 22 C.	6*	23	222	No infection
Ibid. ....	.....	7 days at 5 C.	7*	28	280	No infection
Ibid. ....	.....	7 days at 22 C.	8*	28	300	No infection
Turkey 4..... (Inoculated) Liver lesions	July 10	None. Used at once	10	24	255	Died of inoculated blackhead on 11th day
Ibid. ....	.....	4 days at 5 C.	12	28	310	Died of inoculated blackhead on 14th day
Turkey 12..... (Inoculated) Subcutaneous and liver lesions	July 28	5 days at 5 C.	13*	47	540	No infection
Turkey 10..... (Inoculated) Liver lesions	July 20	None. Used at once	2	44	530	Died of inoculated blackhead on 10th day
Ibid. ....	.....	Frozen for 5 minutes after 12 hours at 1 C.	6*	45	500	No infection
Turkey 20.101..... (Spontaneous) Liver lesions	July 26	5 days at 5 C.	8*	54	730	No infection (The fresh virus was proved virulent by control inoculation)
Turkey 2..... (Inoculated) Subcutaneous and liver lesions	July 31	48 hours at 5 C.	6*	56	730	No infection
Turkey 19-12..... (Inoculated) Subcutaneous lesion	Oct. 9	Frozen for 5 minutes	19-13	104	2,050	No infection (The control inoculation of the opposite breast with untreated virus produced infection)

\* Died of spontaneous blackhead later on, showing turkey to be susceptible to the disease.

for at least 4 days, but it is quickly killed by actual freezing. Whether more resistant forms are present, which are not infective when injected into the tissues of the turkey, or whether such forms are produced elsewhere than in the blackhead lesions, are questions on which these data have no bearing.

## INTRAVENOUS INOCULATION

Turkeys were inoculated intravenously to determine whether certain organs or tissues were more favorable for the development of *Histomonas* than others. Certain of these turkeys also received injections of Niagara blue and India ink, but these dyes did not appreciably affect the results of the inoculation.

There were three objects in injecting these pigments: to stain the lesions vitally; to stain the parasites vitally, and to determine whether these dyes would have any deterrent effect on the growth of the parasite. The lesions were always vitally stained, but in such an irregular manner that no definite conclusions could be drawn as to the origin of the cellular reaction to the virus; the ink distribution was particularly irregular. Niagara blue was present in most of the phagocytic cells in the lesions, and it was possible to demonstrate granules of the dye occupying the same vacuole with one or more *Histomonas*. These granules were often grouped in two masses at the poles of a parasite, but always outside its body. The parasites were never vitally stained, and the presence of the dye in the lesions had no apparent effect on their growth.

Portions of liver or subcutaneous lesions from a freshly killed case of acute blackhead, were ground in a mortar with a small amount of normal salt solution. The resulting suspensions (in doses of 0.5 to 2 c c) were injected into a wing vein, usually the large superficial one extending across the elbow-joint at its inner surface. No immediate effect was noted, except in one instance (No. 30) in which a suspension of lung lesions was employed. This injection produced immediate death, attended by rapid clotting of the blood in the vessels. This phenomenon has already been noted by others in work on poultry.<sup>2</sup> The experiments are briefly tabulated in table 2.

It is apparent from these results that the injection of blackhead virus directly into the blood stream produces disease in only a relatively small proportion of cases, in striking contrast to subcutaneous inoculation, which practically always causes infection. In those cases in which infection follows intravenous inoculation (see table 2, nos. 26, 28 and 29) one or two large initial lesions usually develop in the lungs and smaller secondary lesions distributed in various organs, in order of frequency as follows: lung, liver, kidney, proventriculus, pancreas, small intestine, spleen, cecum and ovary. Occasionally, however, as wide a dissemination of lesions may be associated with either subcutaneous blackhead or the natural disease. The process may extend from the initial lesion of the lung through the thoracic wall, destroy-

<sup>2</sup> Bang, Oluf: *Centralbl. f. Bakteriol.*, I, O., 1908, 46, p. 468.

TABLE 2  
INTRAVENOUS INOCULATION

Turkey	Intravenous Injection *	Findings
7	Aug. 21. 2.5 c c Suspension of liver lesions Aug. 23. 2 c c Suspension of liver lesions (48 hours in refrigerator)	Spontaneous blackhead Aug. 21. Symptoms noted before injected Aug. 26. Died (5th day) no lesions except in caeca and liver (Injections caused no appreciable change in course of disease)
14†	Aug. 6. 1 c c Suspension of liver lesions (Some of suspension escaped into surrounding tissues)	Subcutaneous blackhead Aug. 19. Killed (13th day) Local lesion on wing and secondary lesions in lungs and liver Spontaneous blackhead (early) Both caeca show early lesions
15†	Aug. 6. 1 c c Suspension of liver lesions (Most of suspension escaped into surrounding tissues)	Subcutaneous blackhead Aug. 17. Killed (11th day) Local lesions on wing, numerous small secondary lesions in lungs
16†	Aug. 6. 0.5 c c Suspension of liver lesions	Failed to infect Aug. 20. Killed; normal
17	Aug. 6. 1 c c Suspension of liver lesions Aug. 21. 1.5 c c Thick suspension of liver lesions	Both intravenous injections failed to infect Aug. 28. Breast inoculated with virus Sept. 7. Killed (10th day) Subcutaneous blackhead Local lesion on breast, secondary lesions in lungs and liver (Susceptibility demonstrated)
26	Sept. 9. 2 c c Suspension of liver lesions	Visceral blackhead Sept. 24. Died (13th day) No lesion on wing Extension of process from primary focus in lung through wall of thorax Smaller lesions in lungs, liver, kidney, ovary, pancreas, proventriculus, small intestine and one cecum
28	Aug. 26. 1.5 c c Suspension of liver lesions	Visceral blackhead Sept. 9. Moribund and killed (14th day) No lesion on wing Primary lesion replacing much of right lung Secondary lesions in liver and kidneys
29	Aug. 26. 2 c c Suspension of liver lesions Sept. 9. 2 c c Suspension of liver lesions Sept. 24. 2 c c Suspension of subcutaneous lesion Oct. 8. 2 c c Rich suspension of subcutaneous lesion	Visceral blackhead Oct. 25. Died (17th day after last in- jection) No lesion on wing Primary lesion in lung, extending through thoracic wall with destruction of intervertebral ligament and in- volvement of periosteum Secondary lesions in liver, spleen, kid- neys, proventriculus and duodenum
30	Aug. 28. 2 c c Suspension of liver lesions Sept. 9. 2 c c Suspension of liver lesions Sept. 13. 1 c c Suspension of lung lesion	No infection from first or second in- jection Third injection caused sudden death owing to use of lung tissue
31	Sept. 9. 2 c c Suspension of liver lesions Sept. 24. 2 c c Suspension of subcutaneous lesion Oct. 8. 1.7 c c Thick suspension of subcutaneous lesion	Visceral blackhead Oct. 19. Breast inoculation with virus Oct. 30. Killed (22d day after last in- jection) Subcutaneous blackhead Local lesion on breast Lesions of lungs, pancreas, and cecum of similar size

\* The material used in each intravenous injection, with the exception of those injected on Oct. 8 was proved virulent by control subcutaneous inoculation of other turkeys.

† Injected with Niagara blue and India ink at intervals.

ing muscles, ligaments and other tissues. Although the skeletal muscles are readily invaded, no metastatic lesions have thus far been found in them.

#### IMMUNITY

The frequent failure of intravenous inoculation to produce any evidence of infection led us to consider the possibility of obtaining an artificial immunity by this procedure. It was found, however, that if the intravenous injections were repeated a sufficient number of times, the disease was eventually produced (table 2, no. 29). Furthermore, a negative intravenous inoculation failed to protect the turkey against later subcutaneous inoculation, and the disease resulting from the latter ran its usual fatal course. Intravenous inoculation of a turkey ill with spontaneous blackhead did not appreciably alter the course of this disease or produce additional lesions.

There is little doubt that recovery sometimes occurs in natural blackhead, notwithstanding its usual fatal course. Turkeys showing all the characteristic signs of blackhead and derived from flocks in which blackhead was present have been known to recover. One turkey (no. 25) which showed weakness, loss of weight and sulphur droppings late in November, at the age of 5½ months, improved later on and when killed on Dec. 22, had healing lesions in one cecum and scattered depressions with some scar tissue in the liver.

The immunity in such a recovered case of spontaneous blackhead has not been tested as yet by subsequent subcutaneous inoculations. That immunity follows recovery from blackhead is indicated, however, by the failure of a subcutaneous inoculation of active virus to infect a turkey (43) which had recovered (whether or not because of special treatment) from inoculated blackhead.

The occurrence of a small localized cecal lesion and a few liver lesions in a turkey killed late in Dec., which had not shown any symptoms of blackhead, suggests the possibility that slight attacks may pass unrecognized. The disease, however, may prove fatal even in mature birds. We have recently received a report of the disease occurring in several adult hen turkeys, two of which died. The liver of one of these submitted for examination showed characteristic lesions of blackhead. A year old male said to have lost weight and shown sulphur droppings at about the same time, now appears normal. It is thus quite apparent that those turkeys which fail to become infected in early life may, contrary to popular belief, later on develop blackhead. It is probably true, however, that resistance increases with age so that the outlook is better both in respect to the frequency of blackhead and the prognosis of the cases that occur.



## EMETIN TREATMENT

The efficiency of emetin in the treatment of entamebiasis of man suggested its use in this disease. Of three turkeys 72 days old, inoculated on Sept. 24 with bits of an active subcutaneous lesion, 2 received subcutaneous injection of emetin hydrochloride at intervals, while the third served as a control. Emetin treatment was commenced in one turkey (43) on the day following inoculation and was repeated at intervals of 24 or 48 hours, the dose being sufficient to cause loss of appetite and weakness. A total of 9 mg. of the drug was given in 10 injections during a period of 11 days. This treatment did not prevent the development of the disease, the local lesion developing to considerable size— $2.5 \times 1.5 \times 1.0$  cm.—but later regressed, and the turkey was shown by subsequent subcutaneous inoculation to be immune to blackhead. A similar course of treatment was commenced in the other turkey (42) 3 days after inoculation. The disease was not appreciably affected in this case, death occurring 14 days after inoculation. The control or untreated turkey (41) on account of weakness, was killed on the 11th day, and showed typical inoculated blackhead. It is evident that early sublethal doses of emetin hydrochloride fail to prevent the development of subcutaneously inoculated blackhead. While the recovery of one of the treated turkeys may have been due to the treatment, this isolated instance would not warrant such a conclusion at present, for one of the turkeys inoculated earlier in the season showed a spontaneous regression of the disease when killed.

## SUBCONJUNCTIVAL INOCULATION

To ascertain whether flagellated forms of *Histomonas* escape from lesions in a readily accessible mucous membrane, a turkey (21) was inoculated beneath the conjunctiva near the anterior angle of the eye. Eight days after the inoculation there was a local swelling composed of opaque, whitish tissue beneath the conjunctiva, accompanied by a watery discharge from the eye. The swelling of the subconjunctival tissues increased and the eyeball was gradually covered by the edematous nictitating membrane. There was no ulceration up to the 12th day, when a slight abrasion appeared, possibly due to rubbing the affected side of the head against the feathers. Careful microscopic examination of the discharge showed an occasional rounded organism, but none that assumed the appearance of an active flagellate. Scrapings of the lesion after killing the turkey showed large numbers of *Histomonas* present.

## ATTEMPTS TO PRODUCE BLACKHEAD BY INTESTINAL ROUTE

With the view of determining the source and distribution of the blackhead parasite, apart from the lesions of the disease, various materials were fed and injected.

*Ingestion of Virus.*—Since the alimentary tract is always primarily affected in the naturally acquired disease, it might be expected that feeding of the lesions would result in infections. Three turkeys 2 weeks old were fed repeatedly with fresh subcutaneous, liver and lung lesions, but none developed blackhead as the result of this procedure. One of these on two occasions was placed in a cold room and was thoroughly chilled by partial immersion in ice-water on the first day for four minutes and on the following day for 8 minutes. This turkey apparently thrived on the treatment and grew faster than any of the others hatched from the same setting.

*Injection of Virus into Cecum.*—That the virus, under normal physiologic conditions, might be destroyed in passing through the alimentary tract before reaching the ceca, was considered as a possible basis for the failure to infect by feeding fresh lesions. Accordingly, laparotomy was performed on 3 turkeys and a suspension of ground subcutaneous and lung lesions was injected directly through the wall of the cecum into the lumen. Precautions were taken to avoid undue trauma of the cecal wall, and the introduction of the virus into the tissue. In no instance did the injection of such virus alone result in infection.

That some form of *Histomonas*, not found in acute lesions, might be necessary for the penetration of the cecal mucosa before the natural infection could be initiated, was considered a possibility. It might be expected that such forms would occur in the cecal "core," which is of the nature of a cast of the cavity of the cecum, being composed chiefly of exudate deposited in successive layers. Accordingly, a suspension of ground-up cecal "core" taken directly from a freshly killed case of blackhead, was injected into the ceca of two turkeys, but in neither did blackhead develop.

Partial or temporary obstruction of the cecum, as suggested by the large cores frequently found in spontaneous blackhead, was simulated by injections of melted paraffin (melting-point 42 C.) into the lumen. One turkey received an intracecal injection of paraffin and after a brief illness discharged this material in the form of a cast of the cecum. Two turkeys were given an intracecal injection of paraffin followed by an injection of virus. The larger of these (83 days old) showed

no evidence of infection as the result of the procedure. The smaller turkey, 30 days old (weighing 280 gms.), developed blackhead. However, as the initial lesion in the cecum appeared at the point of puncture, it seems probable that infection resulted from the introduction of virus into the tissue where the cecal wall was punctured. It would not be safe to conclude that obstruction was the determining factor in this single positive case for the delicacy of the ceca in so small a turkey makes it almost impossible to inject into the lumen without some leakage.

*Ingestion of Heterakis Ova.*—In preparation for this experiment, turkeys hatched in an incubator were transferred to a freshly white-washed brooder. Attached to the brooder was a cage, which, except for the first two or three days, was covered with mosquito netting to keep out flies and other insects. Owing to cold weather and the failure of the brooder lamp, many of these specially hatched turkeys succumbed before they could be utilized. We were obliged therefore, to employ smaller groups than was originally intended, and to include for one group, older turkeys that had been fed blackhead lesions previously. On Sept. 22nd the turkeys were separated into 3 groups, each group being placed in a screened coop on clean grass:

Group A: Two turkeys (48 and 49, hatched Aug. 20) were fed large amounts of fresh blackhead lesions on three different occasions (Sept. 22, 24 and Oct. 5). These turkeys, although exposed to cold, dampness and accumulating filth, remained normal up to Nov. 11, and were then used for other experiments, and subsequently proved to be susceptible to inoculated blackhead. At necropsy they showed no evidence of cecal disease.

Group B: Three turkeys (50, hatched Aug. 20, and 51 and 52, hatched Aug. 31) were fed ripe ova of *Heterakis papillosa* on Sept. 22 and 24. One died of blackhead in 18 and 2 in 19 days after the first feeding of *Heterakis* ova, and each showed typical lesions of spontaneous blackhead.

Group C: This group consisted of 3 older turkeys, hatched Aug. 3, that had been fed blackhead lesions without apparent result. One of these (47, that had failed to show the normal weekly increase in weight) was killed on Sept. 24 and showed no lesions of blackhead and no *Heterakis* present. The other two (45 and 46) were fed ripe *Heterakis* ova and blackhead lesions on Sept. 24 and Oct. 5. One died of blackhead in 22 days and the other in 23 days after the first feeding of *Heterakis* ova.

The results of this experiment confirm those of Graybill and Smith,<sup>3</sup> and indicate clearly that a disease in every respect identical with spontaneous blackhead may be experimentally produced by feeding the ripened ova of *Heterakis papillosa*. Apparently it is not necessary, under ordinary conditions, to furnish blackhead virus in addition to the worm ova, for brooder turkeys (Group B) placed on clean ground, without access to insects, contracted the disease when fed the worm eggs alone. It must be noted, however, that the precautions for the comparative isolation of these young turkeys from the time of hatching, did not prevent the appearance of protozoa in their cecal discharges. Although the brooder had been thoroughly whitewashed, the young turkeys which succumbed to cold and exposure showed myriads of intestinal flagellates, chiefly *Tetratrichomonas gallinarum*<sup>4</sup> with fewer *Eutrichomastix gallinarum*.<sup>4 5</sup> Several also showed numerous oöcysts of *Eimeria avium*.<sup>6</sup>

Whether or not the material containing *Heterakis* ova represents a source of the blackhead virus under the given circumstances may possibly be answered by more carefully conducted experiments. On account of the lateness of the season few newly hatched (and hence presumably clean) turkeys were available for subsequent experimentation.

Two of these were fed ripe *Heterakis* ova on Oct. 11, the day after they were hatched. They were then transferred to a clean chamber which served as a brooder, and were provided with sterilized food, grit and water. One of these, a weakling, died 10 days after the ingestion of *Heterakis* ova. Necropsy showed no evidence of blackhead and strange to say, no worms. Both ceca showed rather soft, friable cores which were found on microscopic examination to be composed of fibrin, cellular exudate and bacteria. A careful examination failed to reveal any protozoa.

The other turkey remaining apparently normal, was again fed *Heterakis* ova on Oct. 22 and 23. On account of increase in size it became necessary to remove him from the above chamber and on Nov. 1 (19 days old), he was placed with other slightly younger turkeys in a brooder outdoors. This turkey now failed to gain in weight and was killed on Nov. 8. There was a core in one cecum, the walls of both ceca were somewhat thickened, and numerous ill-defined,

<sup>3</sup> Jour. Exper. Med., 1920, 31, p. 647.

<sup>4</sup> Martin, C. H., and Robertson, M.: Quart. Jour. Micr. Sc., 1911, Part 1, 57, p. 53 and p. 58.

<sup>5</sup> Kofoïd, C. A., and Swezy, O.: Proc. Am. Acad. Arts and Sciences, 1915, 51, p. 289.

<sup>6</sup> Silvestrini and Rivolta: Giorn. Anat. Fisiol., 1873.

small, grayish lesions were present in the liver, which proved to be blackhead on histologic examination. Only 2 immature worms were found on careful search of the cecal contents.

A control turkey, hatched Oct. 12, and kept in the outdoor brooder, received a single dose of the same *Heterakis* material on Oct. 22. This bird died on Nov. 5, of blackhead at a much more advanced stage than was found in the previous turkey, which received its last dose of *Heterakis* ova on Oct. 23, and was killed Nov. 8. Forty-two worms were counted, without a very complete search, from one cecum of the control turkey.

While the result is not wholly conclusive, the early stage of the disease seen in the "clean chamber" turkey strongly suggests that blackhead was contracted after the bird had been transferred to the outdoor brooder. It is a peculiar fact that only two worms developed in the "clean chamber" turkey, which received 3 doses of *Heterakis* ova, whereas the control turkey showed 42 worms in one cecum alone as the result of a single feeding of the same *Heterakis* material.

The subsequent feeding of *Heterakis* ova to several other turkeys resulted in the production of blackhead. The data of all the *Heterakis*-fed turkeys are collected in the accompanying table.

The attempt to eliminate all sources of blackhead infection other than the *Heterakis* material has not yielded definite results, so that it will be necessary to repeat this experiment.

#### INOCULATION TEST FOR PRESENCE OF HISTOMONAS

In order to test the possible infectiousness of various materials, including certain intestinal protozoa and *Heterakis* material, a series of turkeys was inoculated subcutaneously or intramuscularly.

*Intestinal Protozoa.*—Cecal contents of turkeys containing various species of protozoa were diluted with salt solution and inoculated into the breast. There were usually two or more species of protozoa in each cecal discharge used, but as a number of samples were employed, all the known cecal parasites of the turkey, with the exception of *Trichomonas eberthi*<sup>4</sup> were available. The turkeys inoculated with cecal material invariably showed toxic symptoms during the first 24 hours after inoculation. There was local swelling at the site of inoculation, but this subsided in all instances without ulceration, and in no case did blackhead develop. The following protozoa were present in great numbers in the various samples injected: *Tetrachilomastix*

TABLE 3  
FEEDING OF HETERAKIS OVA

Experiment	Turkey	Hatched	Treatment	Result
Group A Fed blackhead	48	Aug. 20	Sept. 22. Fed subcutaneous and lung lesions Sept. 24. Fed lung lesions	Remained normal Nov. 26. Died (Inoculated blackhead of Nov. 11)
	49	Aug. 20	Oct. 5. Fed subcutaneous and lung lesions	Remained normal Dec. 20. Killed (Inoculated blackhead of Dec. 11)
Group B Fed ripe Heterakis ova	50	Aug. 20	Sept. 22. Fed Heterakis ova collected Sept. 7 from chickens Sept. 24. Fed Heterakis ova collected Sept. 10 from chickens	Cecal infection Oct. 5. Sulphur droppings Oct. 11. Died Blackhead lesions left cecum and liver 125 immature Heterakis
	51	Aug. 31		Cecal infection Oct. 10. Died Blackhead lesions left cecum and liver 42 immature Heterakis
	52	Aug. 31		Cecal infection Oct. 11. Died Blackhead lesions left cecum and liver 128 immature Heterakis
Group C Fed blackhead and ripe Heterakis ova  (Group C had previously been fed blackhead lesions on Aug. 17, 18 and 19)	45	Aug. 3	Sept. 24. Fed fresh blackhead lesions (subcutaneous and Heterakis ova collected Sept. 10 from chickens)	Cecal infection Oct. 8. Sulphur droppings Oct. 17. Died Blackhead lesion right cecum and liver (Secondary mycosis) 73 immature Heterakis
	46	Aug. 3	Oct. 5. Fed fresh blackhead lesions (lung and subcutaneous and Heterakis ova collected Sept. 10 from chickens)	Cecal infection Oct. 8. Sulphur droppings Oct. 16. Died Blackhead lesions left cecum, liver and kidney 18 immature Heterakis
Control for Group C	47	Aug. 3	None	Control Sept. 24. Killed No lesions No Heterakis found
"Clean chamber"	58	Oct. 10	Oct. 11. Fed Heterakis ova collected Sept. 7 from chickens	No infection Oct. 21. Died Both ceca contained delicate cores. Microscopically no evidence of blackhead No Heterakis found
	59	Oct. 10	Oct. 11. Fed Heterakis ova collected Sept. 7 from chickens Oct. 22. Fed same material Oct. 23. Fed same material Nov. 1. Moved to outdoor brooder	Cecal infection (early) Nov. 8. No gain in weight. Killed Blackhead lesions both ceca and liver 2 immature Heterakis
Control for No. 59	60	Oct. 12	Oct. 22. Fed Heterakis ova collected Sept. 7 from chickens	Cecal infection Nov. 5. Died Blackhead lesions both ceca and liver 42 immature Heterakis (one cecum)
	63	Oct. 12	Nov. 18. Fed Heterakis ova collected Sept. 10 from chickens	Cecal infection Dec. 1. Sulphur droppings Dec. 4. Killed Blackhead lesions both ceca and liver 87 immature Heterakis

gallinarum,<sup>4, 7</sup> *Eutrichomastix gallinarum*,<sup>4, 5</sup> *Tetratrichomonas gallinarum*,<sup>4</sup> *Entamoeba gallinarum*,<sup>8</sup> *Pygolimax gregariniformis*,<sup>8</sup> and *Eimeria avium*.<sup>6</sup>

*Contents of Diseased Cecum*.—This material was collected from a case of blackhead by searing through the wall of the diseased cecum in order to avoid contamination with *Histomonas* from the infected tissue. The injection on two separate occasions of such material into the breast of a normal turkey failed to produce blackhead.

*Cecal Mucosa*.—The subcutaneous injection of scrapings of the cecal mucosa of several old turkeys also resulted negatively.

*Heterakis*.—*Heterakis* freshly obtained from cases of blackhead were washed several times in salt solution and injected subcutaneously into a turkey with negative result.

*Heterakis Ova*.—Material containing ripe *Heterakis* ova, which when fed to turkeys produced blackhead, was injected into the breast of a normal turkey with negative result.

*Cultures of Ameba and Flagellates*.—Cultures obtained from the *Heterakis* material containing a species of ameba and a flagellate not yet classified, but belonging to the *Bodonidae*, were also injected into turkeys with negative result.

*Tracheal Washings*.—The washings from the trachea in a case of inoculated blackhead with extensive lung involvement, were inoculated subcutaneously without effect.

*Sequestrum*.—In order to determine to what extent *Histomonas* survives in the necrotic material in a subcutaneous lesion a turkey was inoculated with a bit of the necrotic portion. No infection resulted. Later the sequestrum and surrounding tissue from a healing subcutaneous lesion was implanted in the breast of a normal turkey with negative result.

Table 4 gives the data of these various experiments.

It is possible that blackhead virus, even if present, might have been incapable of developing in the presence of the acute inflammatory reaction resulting from the injection of the associated material. Unfortunately the control, that is, the inoculation of a mixture of blackhead virus and cecal contents, was omitted, but will be carried out in future work.

<sup>7</sup> La Fonseca: *Brazil Medico*, 1915, 29, p. 281.

<sup>8</sup> Tyzzer, E. E.: *Jour. Med. Res.*, 1920, 41, p. 199.

TABLE 4  
INOCULATION EXPERIMENTS

Tur- key	Hatched	Injection		Result
		Date—Dose	Material	
2	June 7	June 23 1 c c left breast	Scrapings cecal mucosa from an old turkey	No reaction
3	June 7	June 23 1 c c left breast	Ibid.....	No reaction
7	June 7	July 30 1 c c left breast	Ibid.....	No reaction
20	June 16	Aug. 13 1 c c right and 5 c c left breast	Cecal contents..... <i>Tetrachilomastix gallinarum</i> <i>Eutrichomastix gallinarum</i> <i>Entamoeba gallinarum</i>	Acute inflammation Healing without ulceration
21	June 16	Aug. 13 5 c c right and 1.5 c c left breast	Cecal contents..... <i>Eutrichomastix gallinarum</i> <i>Entamoeba gallinarum</i> <i>Pygollmax gregariniformis</i>	Extensive induration involving muscle Healing without ulceration
22	June 16	Aug. 14 5 c c right and 1 c c left breast	Cecal contents..... <i>Tetrachilomastix gallinarum</i> <i>Elmeria avium</i>	Slight local inflammation
23	June 16	Aug. 17 5 c c right and 1 c c left breast	Cecal contents..... <i>Eutrichomastix gallinarum</i> <i>Entamoeba gallinarum</i>	Local inflammation Healing without ulceration
		Sept. 13 0.1 gm. left breast	Necrotic material from subcu- taneous lesion	No reaction
24	June 16	Aug. 20 5 c c right and 1 c c left breast	Cecal contents..... <i>Tetratrichomonas gallinarum</i> Few <i>Eutrichomastix galli-</i> <i>narum</i>	Slight local reaction Healing within 3 days
		Oct. 11. 3 separate in- oculations in left breast	Washed <i>Heterakis</i> ..... Fresh from diseased ceca 3 cases of blackhead	Slight foreign-body reaction at site of each inoculation
		Oct. 11 1 inoculation right breast	Washed <i>Heterakis</i> Fresh from normal ceca 3 cases of blackhead	
25	June 16	Aug. 21 5 c c right and 1 c c left breast	Cecal contents..... (Blackhead case) <i>Eutrichomastix gallinarum</i> Blastocysts (Possibly a few <i>Tetrachilo-</i> <i>mastix</i> present)	Extensive induration Healing without ulceration
		Oct. 17 3 c c left breast	Cecal contents..... (Blackhead case)	Moderate induration Healing without ulceration Nov. 24. Symptoms of blackhead Nov. 30. Improvement Dec. 22. Killed Spontaneous blackhead Recovery indicated by scars
26	June 16	Aug. 24 0.1 gm. left breast	Sequestrum and tissue from healing subcutaneous lesion	No reaction Susceptibility shown by subse- quent inoculation (table 2)
48	Aug. 20	Oct. 14. 5 c c right, 1 c c left breast	Cultures of amoebae and flag- ellates from <i>Heterakis</i> ma- terial	No reaction
		Oct. 25 2 c c right breast	Washings from trachea of case of inoculated blackhead	No reaction
49	Aug. 20	Oct. 14 1 c c left breast	<i>Heterakis</i> ova..... Collected from chickens Sept. 10	No reaction Same material produced black- head when fed to young turkeys



ASSOCIATION OF *HETERAKIS PAPILLOSA* WITH SPONTANEOUS  
BLACKHEAD

This worm appears to be very generally distributed in common fowls and turkeys. Absent in newly-hatched stock, the prevalence of these parasitic worms depends on the environment in which the birds are reared. Turkeys have been kept entirely free from worms for several months after hatching, by isolation on clean grassed-over areas. When reared on ground previously occupied by turkeys, or when allowed access to hen-coops, young turkeys soon acquire considerable numbers of these worms. It is probable that the warmth of the summer months favors the development of the *Heterakis* ova so that greater infestation occurs as the season progresses. During the past summer a record was kept of the number of *Heterakis* found in practically all turkeys necropsied.

Blackhead cases appeared spontaneously among the turkeys kept in the "old yard," that is, the enclosure which had been used for the two previous summers for experimental work on blackhead. The majority of the cases (8) occurred in July and August, but there were also 2 cases in December. None of these cases showed over 23 worms, and in 2 of the earlier cases none could be found on careful search of the entire cecal contents.

A comparison of the number of worms found in our 8 cases of spontaneous blackhead, and 38 turkeys without cecal infection, shows no predominance in the former series. The examination of 14 cases of blackhead from outside sources also showed some in which no worms could be found, others with few, and yet others in which worms were numerous. It is evident that there is no close relationship between the incidence of blackhead and the number of worms present in our series of turkeys.

It appears probable that a pathologic condition is necessary for the production of blackhead, a condition which may result not only from overwhelming invasion by *Heterakis*, but also from other, at present, unknown causes. We have noted rudimentary casts or cores in the ceca of young turkeys in which there was microscopically no evidence of blackhead, but it is possible that such conditions, with *Histomonas* present in the cecum, may lead to the invasion of the tissues by this organism. The histologic study of such cores, developing in the absence of blackhead, demonstrates their pathologic character—since they consist chiefly of a fibrinous and cellular exudate intermingled with bacteria, but occasionally may also show red blood corpuscles. On

cross-section their laminated structure indicates that they are formed by successive deposits of exudate. The mucous membrane may show no more than slight infiltration and degeneration of the surface epithelium in such instances.

#### EXPOSURE TO COMMON FOWLS

One turkey was kept in the care of a common hen from the time of hatching on September 22. When 18 days old it was placed in a hen-yard for a short time each morning, and when 54 days old it was kept constantly with a small flock of hens. Symptoms of blackhead appeared 13 days later at the age of 67 days. Considerable resistance was shown by this turkey, as it lived 14 days after showing symptoms. Dying at the age of 83 days, chronic blackhead lesions were found at necropsy. *Heterakis* was present in greater numbers (279) in this bird than in any of the turkeys to which the parasite had been fed experimentally. A large proportion of the worms were fully matured. This indicates that young turkeys may gradually acquire many times the number of *Heterakis* than would certainly suffice to produce blackhead when fed experimentally on a single occasion. The result of this experiment also confirms our previous findings as to the danger of exposing young turkeys to the haunts of common fowls. Isolation thus appears to furnish the only practical means at present available of limiting blackhead.<sup>9</sup>

#### EXPOSURE TO TURKEY DROPPINGS OF PREVIOUS SEASON

Three turkeys 9 days old were confined in a cage for 47 days on the accumulated droppings and soil of the night-quarters of the previous season's flock. The attempt was made to exclude flies and insects from this cage by screening but, on the contrary, the cage proved itself a very effective fly-trap on warm days, catching swarms of blue-bottle flies on which the young turkeys gorged themselves. Although this cage became exceedingly filthy, so that the turkeys were at all times dirty and bedraggled in appearance, a much more rapid gain in weight was made than in a control turkey which was hatched on the same date and had the range of a large yard of clean grass. The phenomenal growth of these turkeys closely confined in filthy quarters is somewhat surprising, but may be accounted for by the abundance of insect food available.

<sup>9</sup> Tyzzer, E. E., and Fabyan, M.: Commonwealth of Mass., Dept. of Agri., Dept. Bull. No. 15, March, 1921.

#### INOCULATION OF THE TURKEY EMBRYO WITH BLACKHEAD VIRUS

Four turkey eggs which had been incubated for 12 days were inoculated with active blackhead virus, 2 through the upper and 2 through the lower surface, with the intention of inoculating the amniotic and yolk sacs. The examination of 3 of these, 6 days after inoculation showed one to be sterile, one with a dead embryo, and the third with a living, apparently normal embryo. The fourth egg, on further incubation, failed to hatch, but when opened showed a full-sized, normally developed, turkey chick. In none of these eggs was there any evidence of blackhead.

#### SUSCEPTIBILITY OF OTHER SPECIES TO BLACKHEAD

As stated in a previous paper,<sup>1</sup> it was found possible to infect newly-hatched chickens by inoculation. The local lesions were self-limited, although in one instance there were secondary lesions in the lungs. It was also demonstrated that a certain proportion of pigeons (30%) were susceptible to the subcutaneous inoculation of blackhead. The lesions regressed, however, after the ninth or tenth day.

An attempt has been made this season to infect other species by inoculation. Many European sparrows were inoculated, but without result. Two ducks (Indian runners) were inoculated without result.

Three guinea-chicks were inoculated; local lesions developed in each one, but regression followed after the 12th day. Microscopic examination of the lesions showed extensive cellular reaction, moderate numbers of parasites, but no necrosis.

One pheasant was inoculated when 39 days old. A small local lesion developed slowly and persisted until the bird was killed on Sept. 7, 17 days after inoculation. The local lesion measured 1.3x0.5x0.5 cm. and showed microscopically a marked cellular reaction and numerous parasites, but no evidence of necrosis. No other lesions were noted.

#### SUMMARY

The virus of blackhead, as found in turkey liver lesions, survives for at least 4 days at 5 C., deteriorates more rapidly at 22 C. and is immediately destroyed by freezing.

As compared with subcutaneous inoculation, intravenous inoculation results much less frequently in infection. The failure of an intravenous inoculation to cause infection is not due to an insusceptibility of the turkey employed; for if this procedure is repeated a

sufficient number of times, infection follows. Furthermore, turkeys that have been inoculated intravenously with negative result may now be successfully inoculated subcutaneously. That intravenous inoculation should so frequently fail to produce infection is the more remarkable since dissemination of the virus by the blood stream almost invariably occurs in infected turkeys.

These results clearly show that immunity is not produced by negative intravenous inoculation; it has been found impossible, however, to infect a turkey that has recovered from inoculated blackhead. Although not yet demonstrated, it may be expected that turkeys recovering from spontaneous blackhead will also prove immune. Resistance to blackhead seems to increase with age, so that fewer maturing birds show symptoms of infection and recovery is evidently more frequent. It is not improbable that mild attacks may pass unrecognized in large turkeys.

The treatment of the turkey by repeated subcutaneous injections of emetin hydrochloride in doses sufficiently large to produce toxic symptoms, fails to prevent the development of subcutaneous blackhead, even though commenced immediately after inoculation. Of two cases thus treated, one recovered; but spontaneous recovery from blackhead occasionally occurs.

Repeated injections of Niagara blue and India ink (whether before or after inoculation), do not apparently alter the course of the disease.

The study of the conjunctival discharge in subconjunctival blackhead furnishes no evidence that *Histomonas* escapes from a mucous surface in flagellated form. This is consistent with the findings in previous histologic studies of cecal lesions in which no intermediate stages were found between *Histomonas* and any of the flagellates in the cecal glands or contents.

Attempts to produce blackhead by the intestinal route show that the disease is not caused by the mere feeding of infected tissues. The injection into the lumen of the normal cecum of either virus from active lesions, or the substance of "cores" from ceca infected with blackhead, may not result in blackhead. Temporary obstruction has been experimentally produced by the injection of melted paraffin into the lumen of the cecum. This alone, or in conjunction with blackhead virus, fails to cause cecal infection.

The feeding of large numbers of ripe ova of *Heterakis papillosa* to isolated groups of turkeys has resulted in typical blackhead. It was noted, however, that turkeys raised in fairly strict isolation acquired

great numbers of intestinal protozoa before leaving the brooder. Apparently ripe *Heterakis* ova may fail to develop when fed to young turkeys kept in a clean chamber and furnished with sterilized food and water, but they readily develop in those having access to the soil. The reason for this is not apparent.

The subcutaneous inoculation of various materials—cecal discharges containing various species of cecal flagellates and amebae; blastocytes, cultures of protozoa of cecal origin, exudate from a cecum showing blackhead lesions, ripe *Heterakis* ova and washed *Heterakis* from several cases of blackhead—have all failed to produce blackhead. It is possible that the acute inflammation resulting from the injection of cecal contents may interfere with the development of *Histomonas*, were the latter present in the material used. Although this organism is sometimes found in considerable numbers in the air-spaces, in stained sections of pulmonary lesions of inoculated turkeys, the injection of tracheal washings from a single case resulted negatively. The results of the inoculation of necrotic portions of subcutaneous lesions, or material from healing lesions indicate that *Histomonas* does not persist long in dead tissues.

While blackhead appears to develop quite constantly as the result of feeding ripe *Heterakis* ova, the study of a large number of turkeys indicates that the disease may occur when this worm is rare or entirely absent from the ceca. It is also apparent that young turkeys do not develop blackhead as readily from the slow acquisition of *Heterakis* ova, as they do from large overwhelming doses, such as those given experimentally. These findings suggest that the invasion of the tissue by *Histomonas* in spontaneous blackhead is not dependent solely on the presence of *Heterakis* but on pathologic conditions that may occur not only in association with this parasite worm but also quite independent of it.

Soil contaminated with the droppings of turkeys of the previous season may not of itself furnish the conditions which would cause young turkeys kept thereon to develop blackhead.

A self-limited infection, similar to that produced in young chickens and pigeons, follows the inoculation of blackhead into young pheasants and guinea-chicks. All of these species show a much more extensive tissue reaction to the virus than that which occurs in the more susceptible turkey. Both the European sparrow and Indian Runner duck appear to be nonsusceptible.

The turkey embryo is apparently unsuitable as a medium for the development of *Histomonas*: for incubated turkey eggs were inoculated with blackhead virus without result.

Blackhead has followed the exposure of a young turkey to common fowls, confirming the experience of the previous years and emphasizing the importance of isolation in rearing turkeys.

# A RAPID METHOD OF DETERMINING THE PRESENCE AND TYPE OF BOTULINUS TOXIN IN CONTAMINATED FOODS \*

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The importance of an early diagnosis of botulism like that of tetanus and diphtheria is emphasized by the fact that the benefits of antitoxin depend on early administration. In the case of botulism this is confirmed by clinical reports and experiments. Thus, Forssman<sup>1</sup> concludes from his work on guinea-pigs and rabbits that botulinus antitoxin, to have marked therapeutic effects, must be given before the appearance of respiratory symptoms. Kempner,<sup>2</sup> Dickson and Howitt<sup>3</sup> and others, however, have shown that it is possible to save guinea-pigs by the injection of botulinus antitoxin as long as 24 hours after the injection or feeding of a fatal dose of toxin (fatal in 48 hours), even though definite symptoms of intoxication were manifest.

There are at least two distinct types of botulinus toxin, known as A and B. The antitoxin prepared against each type is specific for the homologous toxin and will not protect against the heterologous toxin. These facts clearly indicate the importance of using both types of antitoxin provided the type specific for the toxin causing the poisoning is not known.

The problem at hand then includes, in addition to an early diagnosis, the type determination of the infecting organism.

Graham and Schwarze<sup>4</sup> suggest the feeding of the responsible food to chickens as a preliminary method of determining the type strain of *B. botulinus*. They found that type A is fatal when fed to mature

Received for publication, May 16, 1921.

\* In accordance with the nomenclature recommended by the Society of American Bacteriologists (J. Bacteriol., 1920, 5, p. 191), *Bacillus botulinus* becomes *Clostridium botulinum* (van Ermengen).

\* This is part of an extensive investigation on food poisoning, with special reference to botulism, that is being done under the auspices of the Advisory Committee on the Toxicity of Preserved Foods of the National Research Council under a grant from the National Cannery Association.

<sup>1</sup> Centralbl. f. Bakteriöl., I. O., 1901, 29, p. 541.

<sup>2</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1897, 26, p. 481.

<sup>3</sup> Jour. Am. Med. Assn., 1920, 74, p. 718.

<sup>4</sup> Jour. Infect. Dis., 1921, 28, p. 317.

chickens while type B does not produce illness in chickens even when fed in liberal amounts. In results to be published elsewhere I have found that it is possible to produce botulism in chickens by feeding foods infected with type B as well as with type A. At least one outbreak of limberneck in chickens reported by Dickson<sup>3, 5</sup> was shown to be caused by *B. botulinus*, type B. This outbreak occurred at Berkeley, Calif., during 1918. From these observations it is apparent that the method of Graham and Schwarze of typing *B. botulinus* by the feeding of chickens cannot be accepted.

During the course of work in this laboratory I have found that the intraperitoneal injection in white mice of at least 100 M L D of botulinus toxin produced symptoms of botulism usually in from 2 to 4 hours and death follows the appearance of symptoms within from 1 to 2 hours. It occurred to me that advantage could be taken of this observa-

TABLE 1

TOXIN 23, TYPE A: VARIATION OF LENGTH OF INCUBATION PERIOD WITH VARIABLE AMOUNTS OF TOXIN; M. L. D.—0.0001 c c

Weight of Mouse in Grams	Amount Injected in C c	Number M L D	Results
15	0.3	3,000	Mouse died within 2 hours and 10 minutes
15	0.1	1,000	Mouse died within 4 hours and 25 minutes
14	0.03	300	Mouse died within 4 hours and 25 minutes
16	0.01	100	Mouse died within 6 hours and 30 minutes
14	0.003	30	Mouse died within 7 hours
15	0.0003	3	Mouse died within 24 hours

tion to determine the presence and also the type of toxin within a few hours. The usefulness of the test, however, is dependent on the possession of the infected food and also on the potency of the toxin in this food. The weaker the toxin the longer the time necessary to obtain the results, owing to the longer period of incubation in mice. Table 1 shows the variation in the length of the incubation period when injecting mice with variable amounts of toxin.

From these results it is evident that the period of incubation in botulism is dependent on the amount of toxin. This corresponds to similar observations on tetanus and diphtheria toxins. It also follows that some idea of the potency of the toxin in infected foods can be obtained from the length of the incubation period in mice which have been inoculated intraperitoneally.

The method of type determination consists in the intraperitoneal injection in each of a number of white mice of about 0.5 c c of the

<sup>3</sup> Jour. Am. Med. Assn., 1918, 71, p. 518.



filtrate of the infected food, some of the mice having been previously injected with type A antitoxin and some with type B antitoxin. If previously immunized mice are not available, it is just as good to mix some of the suspected toxic filtrate with type A antitoxin and some with type B antitoxin, and then inject the mixture into the mice intraperitoneally. Mice of 15 to 20 gm. of weight tolerate 1 c c of non-irritating fluid in the peritoneal cavity very well. If the food contains the toxin of *B. botulinus* type A the mice receiving no antitoxin and those receiving type B antitoxin will die, while those receiving type A antitoxin will survive. On the other hand, if the food contains toxin of the type B organism only those receiving type B antiserum will live. In this way both the presence and the type of toxin may be determined in from 4 to 6 hours.

TABLE 2

Weight of Mouse in Grams	Amount of Filtrate Injected Intraperitoneally in C c	Amount of Antitoxin Injected Intraperitoneally		Results
		Type A	Type B	
10	0.1	.....	.....	Mouse died in 2 hours
25	0.1	.....	.....	Mouse died in 7 hours
12	0.004	.....	.....	Mouse died in 74 hours
11	0.001	.....	.....	Mouse survived
11	0.1	.....	0.1 c c	Mouse died in 4 hours
12	0.01	.....	0.1 c c	Mouse died in 20 hours
14	0.1	0.1 c c	.....	Mouse survived
12	0.01	0.1 c c	.....	Mouse survived

The basis of this method is not original, however, the extremely short incubation period of the toxin when large amounts are injected intraperitoneally in mice has not been previously reported, and it is the application of this observation in the method which seems to make it worth while to report.

From foods such as string beans, spinach, asparagus and olives it is possible to use the accompanying liquor as the material for injection, while with other foods, such as meat, or when liquor is not available, the food should be thoroughly triturated with a small amount of water or salt solution and the resulting liquid used for injection. It is preferable to filter the liquor through a Berkefeld or Mandler filter so as to obtain a sterile filtrate for inoculation.

Table 2 gives the results obtained with the ripe olives which caused the New York outbreak of botulism,<sup>6</sup> and will give some idea of this method of determining the type of *B. botulinus* in infected foods. The olive liquor was filtered through a Mandler filter and the inoculations shown in the table were made.

<sup>6</sup> Sisco, D. L.: Jour. Am. Med. Assn., 74, 1920, p. 516.

From these results it was possible to make a diagnosis of type A infection within a few hours. Ordinarily it is recommended to inoculate some mice with larger quantities, 0.5 to 1.0 c.c. of the suspected filtrate, for the toxin may be present in weak concentration. The approximate strength of the antitoxin must be known so as to be certain that a sufficient amount is used to protect against the toxin injected. In the case mentioned 1 c.c. of antitoxin, type A, protects against about 3,000 M L D of homologous toxin; while 1 c.c. of antitoxin, type B, protects against about 10,000 M L D of type B toxin.

The specificity of the toxin and antitoxin of the two types, A and B, is very distinct, as I have been unable to obtain any protection against a heterologous toxin when using even massive doses of antitoxin. For example, 1,000 units of type B antitoxin (1 unit of antitoxin was arbitrarily set as the amount of antitoxin which would protect a 15 gm. mouse against 1 M L D of homologous toxin) failed to protect a mouse against 10 M L D of type A toxin, while 600 units of type A antitoxin failed to protect a mouse against 10 M L D of type B toxin. There is thus no danger in obtaining cross protection in the type determination tests when using massive doses of heterologous antitoxin. These results again emphasize the importance of using specific type antitoxin in treatment of botulinus intoxication.

# NEGRI BODIES IN THE SALIVARY GLANDS AND OTHER ORGANS IN RABIES

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It has long been recognized that the bite of a rabid animal is the ordinary means of transmitting rabies. Quite naturally, therefore, the saliva was suspected of harboring the infectious agent and early investigators easily proved this to be the case. According to Högyes<sup>1</sup> and others, Zinke<sup>2</sup> is credited with having been the first to produce rabies by introducing saliva from a rabid dog into wounds of another dog, a rabbit and a cock. Following at intervals Gruner,<sup>3</sup> Magendie,<sup>4</sup> Hertwig,<sup>5</sup> Rey<sup>6</sup> and others have simply multiplied evidence of the virulence of the saliva of rabid animals, both carnivorous and herbivorous. Excellent reviews of the literature on this subject are given by G. Högyes, M. Casper,<sup>7</sup> and J. Koch<sup>8</sup> so that any detailed references to it here seem unnecessary. A consideration of the work on this phase of the subject would seem to indicate that the saliva is more likely to be virulent in the case of animals infected with street virus than with fixed virus, and this would also seem to be borne out by the results of my own investigation. Elsenberg<sup>9</sup> reviews carefully the early literature on the anatomic changes in the salivary glands. He believes the process starts as a parenchymatous inflammation and later assumes the character of an exudative process.

Although careful studies have been made of the histology of the salivary glands in rabies both before and since Negri's<sup>10</sup> discovery of bodies in the ganglion cells of the brain of rabid animals, which are now regarded as diagnostic of rabies, only one author has recorded finding anything resembling Negri bodies in these glands, namely,

Received for publication May 19, 1921.

<sup>1</sup> Nothnagel's Spec. Path. & Ther., Lyssa, 1897.

<sup>2</sup> Neue Ansichten der Hundswuth, etc., 1804, p. 180.

<sup>3</sup> Scharfs Arch. de Mediz. Polizey, Bd. 3, 1, Samml., S. 10.

<sup>4</sup> Jour. de Phys. Exp., 1823, 3, p. 382.

<sup>5</sup> Beiträge zur näheren Kenntnis der Wutkrankheit, 1829.

<sup>6</sup> Recueil de med. vet., 1841, 1842, 1856.

<sup>7</sup> Lubarsch-Ostertag: Ergebn. d. Allgemein Path., etc., 1900, 7, p. 662.

<sup>8</sup> Kolle and Wassermann: Handbuch path. Mikro-Org., 1913, 8, p. 832.

<sup>9</sup> Virch. Arch., 1882, 87, p. 89.

<sup>10</sup> Ztsch. f. Hyg. u. Infectiouskr., 1903, 44, p. 519.

Stephanescu,<sup>11</sup> who describes the finding of Negri bodies in the glandular cells of the parotid of a rabid dog. Lina Luzzani<sup>12</sup> says, concerning the examination of the salivary glands in a case of human rabies, "Ich will zuletzt erwähnen dass sowohl in den Speicheldrüsen als in der Narbe der gebissenen Haut das Aufsuchen parasitärer Formen, die denen in den Nervenzellen befindlichen gleich wären, vollständig negativ ausgefallen ist."

Ganslmayer,<sup>13</sup> in the examination of the submaxillary glands from 40 mad dogs, obtained experimentally positive results in 37 of the cases. Positive results were less frequently obtained with parotid and sublingual glands, but in none of the glands did he find anything resembling the Negri bodies in Ammon's horn.

In spite of a full knowledge of the almost uniform failure of microscopic examination of the salivary glands in rabies to confirm the assumption that Negri bodies are the cause of rabies or to suggest some other agent, it was with only a partial appreciation of the difficulties to be encountered that this study of the structures of the mouth and the salivary glands of rabid dogs was undertaken.

Through the kindness of Dr. F. O. Tonney, Director of the Laboratory of the Chicago Department of Health, I obtained the heads of 18 rabid dogs. As controls the salivary glands from 15 normal dogs were examined. The material was fixed by different methods; Zenker's fluid, bichloride of mercury and acetic acid, alcohol and formalin, and the tissue was infiltrated with paraffin. Various staining methods were used—alcoholic eosin and methylene blue, rosanilin and methylene blue, iron hematoxylin, Giemsa and Levaditi stains. As a whole, the alcoholic eosin and methylene blue was found to be most satisfactory.

A microscopic examination was made of the salivary glands of 15, and of the epithelium of the tongue and various portions of the mouth, the pharynx and esophagus, of 9 supposedly normal dogs. In 4 cases some of the larger ducts of the submaxillary gland contained rather numerous ameboid-like bodies, in 2 cases a definite parasite was present in the glands (see p. 302), and in 3 cases the submaxillary glands contained rather large numbers of secretion granules.

It seems best at this point to describe briefly certain bodies found in the salivary glands and epithelium of the mouth in normal dogs for comparison with those found in rabid dogs.

<sup>11</sup> Compt. rend. Soc. de Biol., 1907, 62, p. 886.

<sup>12</sup> Centralbl. f. Bakteriol., I, O., 36, p. 540.

<sup>13</sup> Ibid., 1910, 55, p. 487.

In three cases the submaxillary glands contained considerable numbers of granules which stain bright red with rosanilin and bluish black with iron hematoxylin but not at all or slightly with eosin. These granules occur in the glandular cells and occasionally in the epithelial cells of the ducts. They may be loosely scattered throughout the cells or closely aggregated in cyst-like cavities and are regarded as secretion or zymogen granules. Sections from the other cases contain few

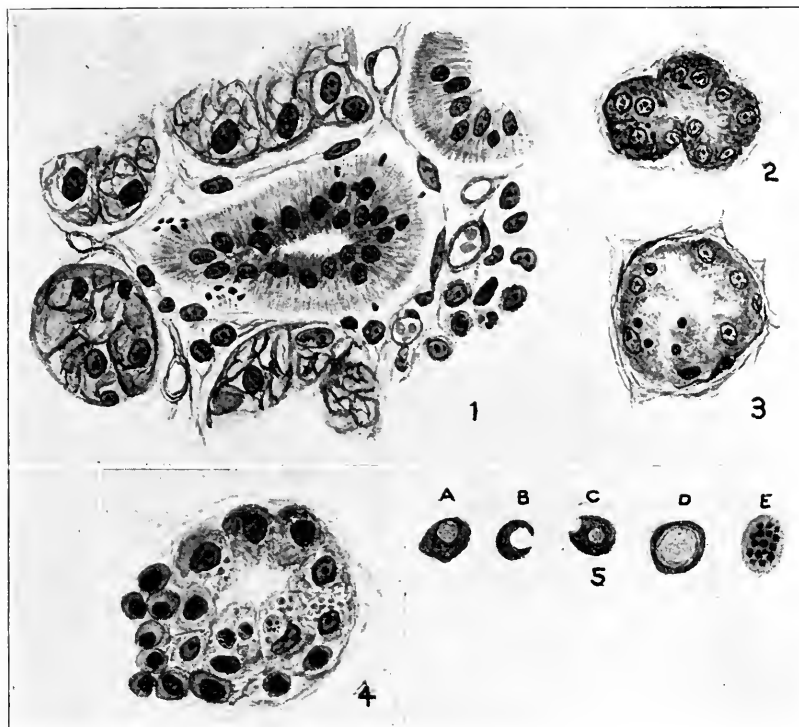


Fig. 1.—Small Negri bodies in the duct of the salivary gland.

Fig. 2.—Small Negri bodies in the acinar cells of the salivary gland.

Fig. 3.—Medium sized nucleated forms of Negri bodies in the acinar cells.

Fig. 4.—Negri bodies associated with undifferentiated bodies in the acinar cells.

Fig. 5.—a, b, c, d, various forms of Negri bodies present in the salivary gland; e, sporogenous form.

or none of the granules. It is easily seen that the presence of such granules would make difficult or impossible the differentiation of and recognition of small, delicately stained Negri bodies.

The ameboid-like bodies found in the ducts of the submaxillary gland of 4 normal dogs were even more frequently found in the rabid animals.

These bodies vary greatly in size, and are usually round or oval, but the larger forms may be quite irregular in shape. They are sometimes quite numerous, particularly the smaller forms. In some cases they appear vacuolated; this is especially true of the larger forms. The vacuoles occupy the central portion, and the peripheral zone presents an homogeneous appearance. Not infrequently these bodies contain deeply stained refractile granules or blue stained areas and often resemble closely certain forms of Negri bodies. The small forms often appear homogeneous and structureless. The ground substance stains pink with eosin and red with rosanilin. For the most part they appear free within the lumen of the ducts, but they are also seen apparently within or between the epithelial cells of the duct or lying closely against them. In the lumen they are usually surrounded with mucous and often with much granular debris. No bodies resembling Negri bodies are found in the ganglion cells lying close to the ducts containing these bodies, as is often the case in the glands of rabid animals.

A very interesting and multiform parasite was present in the salivary glands of two old dogs which were used as controls. The parasite is described in some detail for the reason that certain forms found in these glands resemble somewhat forms found in the glands of rabid dogs but without the accompanying distinctive forms present in these two cases.

These organisms were chiefly confined to the anterior lobe of the submaxillary gland, but in one case certain forms were found in considerable numbers in the parotid. The parasites in the first location are numerous, of large size and very striking in appearance and occur in the acinar cells. A form which probably represents the vegetative stage consists of a deeply stained central portion of variable size surrounded by a clear, unstained zone of varying width. These bodies are usually about 10 mikrons in diameter. The central portion may be more or less irregular in outline, but usually small bodies are seen budding off from it or lying free in the clear peripheral zone. Another form which occurs in about the same numbers consists of a clear, lightly blue stained ground substance in which are imbedded one or several round, deeply red-stained bodies, which usually differ considerably in size. These cyst-like forms vary more in size than those first described. A more detailed description will be given in another article (see p. 302). Associated with the bodies described in one of these cases and quite often seen in the salivary glands of both normal and rabid

dogs are round bodies which stain a light pink with eosin, the central portion taking a deeper stain, and the color shading off toward the periphery without differentiation into central and outer portion. They occur singly or in considerable numbers in the acinar cells of the submaxillary gland. These bodies appear quite homogeneous with no nucleus visible. There is considerable variation in size, although when a number occur in the same cell they are usually of about the same size. With the exception of degenerative changes in the infected cells, there is very little reaction in the tissues. No more highly differentiated forms are associated with these bodies in any of the glands from normal dogs, except the two described.

No changes worthy of mention at this time were found in the epithelium of the esophagus, pharynx, roof of the mouth or of the tongue.

Microscopically the salivary glands of rabid dogs present quite a variety of appearances. In some the changes are slight and chiefly in the region of the ducts, in others there is a moderate but rather uniform distribution of changes throughout the gland. Sometimes only a comparatively small part is involved, while the rest appears normal, or the larger part of a gland may appear greatly altered and a small part be apparently more recently and less severely involved. The infiltration is most marked about the ducts and blood vessels but also penetrates the gland substance. The infiltrating cells are for the most part mononuclears; in some instances there are considerable numbers of polymorphonuclear leukocytes present, but abscess formation was not noted in any of the cases. There is often more or less infiltration of the duct epithelium and some desquamation of the epithelial cells. The amount of degenerative change in the acini varies greatly. In the cases examined the submaxillary gland was much more frequently involved than the parotid.

In one case in which the alterations in the salivary glands were mild and chiefly in the vicinity of the ducts, considerable numbers of small delicately stained bodies are present in the duct epithelium. These bodies are round, fusiform or irregular in shape and usually contain one or more deeply stained refractile granules centrally or eccentrically placed and are especially well seen in the outer portions of the cells. The delicacy of structure and staining make a proper background necessary for their recognition and differentiation from bits of coagulated serum, etc. They doubtless occur in the cells of involved acini but

positive identification is usually impossible on account of the staining reaction of the cells. Similar bodies are present in the ganglion cells included in sections of this gland. These small bodies were occasionally seen in the duct epithelium of other cases. In fact, this form of Negri body was found in the ganglion cells in the salivary glands of eight of the rabid dogs, while nothing resembling them was found in the ganglion cells in this location in normal dogs. These forms correspond to certain forms found in the brain, especially in brains of fixed virus guinea-pigs and are very well described by Williams and Lowden.<sup>14</sup>

In other glands degenerative changes in the acini are much more general, including the greater part of the gland, infiltration being a rather marked feature. Not infrequently the greater part of the submaxillary gland may be thus involved, while a small part of the gland may show less change and a more active process. Careful search in the more changed portion usually discovers small numbers of medium sized rather delicately stained Negri bodies, while in the less changed portions there are considerable numbers of bodies which at least strongly resemble Negri bodies. Some of the acini in this location are apparently normal, but scattered throughout are many acini in which a kind of liquefaction of the cytoplasm seems to have occurred, the lumen being filled with coagulated muco-albuminous material. A few nuclei may usually be seen about the wall of the acinus. There may be comparatively little infiltration in these locations or there may be a considerable amount, the infiltrating cells being of the mononuclear type chiefly. Here, as in the brain, one may find one or more of these bodies in a seemingly normal cell, but usually the cells containing parasites show more or less degeneration and of some only remnants remain. The bodies found here are mostly small and medium sized forms, with usually a blue stained central portion and with or without the refractile granules. Certain forms which I have not found very often in the brains are present in one case in considerable numbers. In these a rather wide deeply red stained rim surrounds a circular, lighter blue or bluish pink stained central portion or sometimes two such lighter portions which appear undifferentiated. The shape of these bodies is irregularly round or oval. One occasionally sees forms in which the central portion seems on the point of extrusion, vacuolated forms are also seen in the same cells with more light bluish-pink stained bodies.

<sup>14</sup> Jour. Infect. Dis., 1906, 3, p. 452.



and one is led to infer that the latter may have been extruded from the vacuolated forms. In this and one other case forms which seem to correspond to Watson's<sup>15</sup> sporogenous forms are present in the acini. These forms vary in size but are quite regularly round or oval and consist of a homogeneous bluish pink stained ground substance or cytoplasm in which are imbedded rather brightly red stained, small, spherical bodies. In some cases these spores are small and lie close together in the center of the parent body so that unless one observes carefully, the real structure of the organism is not appreciated, but in most cases these spore-like bodies are easily differentiated from the more basic staining cytoplasm in which they lie. Quite frequently, especially in sections of the brain, one finds bodies in which only 2 or 3 spores remain within the parent body, while just outside but still in contact with it are a number of bright red stained spore-like bodies. It seems probable that the number of spores is variable, at least I have not been able to make out a definite number. Occasionally there can be seen a weakly blue stained, irregularly oval central part which probably represents the nucleus, but generally no nucleus is visible. The sporogenous forms described by Watson differ somewhat from those described by me. He describes the protoplasm of the parent cell as staining pink with no indication of a nucleus, while in my cases I not infrequently observed bodies in which there were indications of a nucleus, and the protoplasm usually took a decidedly basophilic tint. These differences may, however, be due to differences in the staining method, for in the main the descriptions agree very well, and we are doubtless dealing with the same forms.

Exactly similar forms were found in sections of the brain from two of these cases but no histories of the dogs in which these were found were obtainable. In the cases described by Watson, the course of the disease was unusual. The dogs had presented the usual clinical symptoms: uneasiness, restlessness, evident signs of distress and irritability and during this stage had bitten people. The dogs were consequently confined and about 20 days later died, the reported cause of death being distemper. A somewhat belated examination of the brain, however, disclosed the presence of Negri bodies. These cases would suggest that sporogenous forms develop later in the course of the disease.

I was able to obtain all of the organs from only one rabid dog, and these were not in a good state of preservation; consequently the results

<sup>15</sup> Jour. Exper. Med., 1913, 17, p. 29.

of the examination were not entirely satisfactory. Negri bodies were found in the brain but in none of the other organs except the suprarenal. In the suprarenal these bodies were found in the cells of the medulla, and were chiefly of medium and small size and quite numerous in places and typical in appearance. Many of them have the dark blue nucleus with dark brown refractile granules in the surrounding cytoplasm. There are also many of the tiny undifferentiated forms present. The changes found in the suprarenals agree with those described by Cornwall<sup>16</sup> and consist of a swelling of the gland with more or less autolytic change in the cells. There is no infiltration. The presence of Negri bodies in this location agrees with my observations on the suprarenals in rabid guinea-pigs in which the disease developed slowly. There were only slight changes in the salivary glands, and these were chiefly in the ducts in which a considerable number of the ameba-like organisms were found.

In order to determine whether Negri bodies are present in the mouths of rabid dogs in recognizable forms smears were made of the saliva present in the mouths of some of the dogs examined, also later in the work smears of scrapings from the tongue were examined. Examination of the smears of the saliva has not yielded satisfactory results. The deeply red stained granules described as present in the salivary glands of normal dogs are sometimes seen in large numbers in these smears, but bodies that could be positively identified as Negri bodies were not often found. In two cases examined, smears of scrapings of the tongue were made and numerous bodies, which in some respects resemble Negri bodies, were found. Whether these bodies are identical with the ones found in the epithelium of the tongue under normal conditions, or whether they are bodies carried with the saliva from the ducts and acini of the salivary glands, is difficult to determine as in staining reactions, size and structure all of these bodies so much resemble one another.

In addition to the study of the salivary glands, oral cavity and brains of rabid dogs, an examination was made of the tissues of rabid guinea-pigs. Two of these pigs were inoculated with fixed virus, 3 with the filtered (Maassen) emulsion of the brain of 1 of the fixed virus pigs, and 2 with filtered emulsion of the salivary glands from rabid dogs. All were inoculated by the intracranial method. The 2 fixed virus pigs showed symptoms of the disease and were killed 6 and

<sup>16</sup> Indian Jour. Med. Res., 1919, 7, p. 148.

11 days, respectively, after inoculation. The 3 pigs injected with filtered emulsion of fixed virus brain, from one of the aforementioned pigs, were killed 7 days after injection.

While the study of the brains from these animals added much to my own knowledge of the subject, there is little to add to the already complete literature on the pathology of the brain in rabies. I should like, in this connection, to mention the finding of small bodies in the nuclei of ganglion cells in the brains of the experimental animals especially, which after considerable study I regarded as small forms of Negri bodies. These bodies stain rather brilliantly red, are round, quite distinctly outlined and usually undifferentiated, and the nucleus containing them often shows more or less degeneration. In one case a somewhat larger differentiated form was occasionally seen in this location, which in every respect resembled the smaller nucleated forms found in the cytoplasm of the nerve cells. These bodies are doubtless similar to those seen by Williams<sup>17</sup> in the sections of fixed virus brains.

No lesions or Negri bodies were found in sections of the salivary glands of the guinea-pigs. In one or two cases bodies which resembled certain forms of Negri bodies were seen in the lymph glands and spleen, but certain identification of the bodies in this location is difficult. I am certain, however, that Negri bodies were found in the suprarenal glands of the 2 guinea-pigs living 31 and 41 days after inoculation. These were present in both the cells of medullary and cortical portions of the gland. There were degenerative changes in the cells but almost no infiltration of the tissues. Other organs were apparently negative.

There is now general agreement as to the value of Negri bodies in the diagnosis of rabies and as to their protozoan nature. The complete life cycle of the organism has not as yet been observed, and there are some not very important differences of opinion as to its classification. Negri<sup>18</sup> regarded them as parasites of an animal nature. Calkins<sup>18</sup> thinks *Neurocytes hydrophobiae* offers evidence of rhizopod affinities, the variable forms, uninucleate condition leading to a condition of distributed chromatin, and the budding phenomena being common to rhizopods and not to sporozoa. Williams and Lowden, and Watson place them among sporozoa, but since, according to Hertwig,<sup>19</sup> the sporozoa must be regarded as rhizopods or flagellates modified by parasitism, this can scarcely be regarded as a serious disagreement. Watson still

<sup>17</sup> Jour. Infect. Dis., 1906, 3, p. 467.

<sup>18</sup> Protozoology, 1909.

<sup>19</sup> Manual of Zoology, 1912.

further classifies these organisms, placing them in suborder Cryptocytos or Microsporidia and among the Oligasporagenea of the Glugeidae family.

The character of the organisms found in the salivary glands of normal persons makes it a difficult matter to differentiate between these and the very numerous forms of Negri bodies in the rabid animal. In staining reaction and structure certain forms of these organisms resemble Negri bodies very closely, as has previously been pointed out. To illustrate: In one of these cases of rabies, the larger ducts of the submaxillary gland contain many small oval bodies, and in the ganglion cells lying close to the ducts are many small Negri bodies of about the same size. The latter appeared somewhat less homogeneous than those in the ducts, but the variation was not greater than that which often occurs among Negri bodies in a single section of the rabid brain. That the bodies in the ducts are not Negri bodies seems likely since similar bodies are sometimes found in this location in the normal animal; while the bodies in the ganglion cells are doubtless Negri bodies as bodies like them have not been found in the ganglion cells of the salivary glands of normal dogs.

The same uncertainty is felt in regard to the small, round, lightly pink stained, undifferentiated bodies found associated with Negri bodies in the salivary glands of rabid dogs. These undifferentiated forms are sometimes seen in the same cell with seemingly undoubted Negri bodies in the glands of rabid animals, while quite similar forms are found in the glands of normal dogs, but unassociated with anything resembling Negri bodies. In one or two cases of rabies these round lightly stained bodies seemed to be thrown off from the deeply red stained bodies with lightly blue stained centers, which sometimes appeared to contain a vacuole and at other times seemed on the point of extruding a round lightly stained body. Although not absolutely sure that these red bodies are forms of Negri bodies they so closely resemble forms found in the brains of these animals that they have been considered as forms of Negri bodies. An added reason for believing they may be Negri bodies is the absence of similar forms in normal cases. Apparently the only satisfactory means of answering these questions would be the cultivation of the organism.

In spite of these difficulties there are some things which stand out clearly in a comparison of the rabid glands with those from normal dogs. There is a more extensive involvement of the glands in some

cases of rabies than has been observed in any of the normal animals, also the character of the changes differ in some particulars in cases in which the salivary glands in normal dogs are infected with a certain parasite, the glandular cells of a definite portion, usually the anterior lobe of the submaxillary, are almost literally filled with organisms, and their presence calls forth only a mild reaction in the tissues, whereas in the rabid dog the submaxillary glands may be quite uniformly altered throughout, the changes consisting of rather advanced degenerative changes in the glandular cells and much infiltration, while comparatively few recognizable forms of Negri bodies may be found. In other cases the rabid glands may show mild changes, and considerable numbers of the small delicately stained Negri bodies may be present in the duct epithelium and ganglion cells and seemingly, at least, in less numbers in the glandular cells.

Without doubt, in some cases I have found Negri bodies associated with organisms that occur in these glands in the normal dog, in which cases it was impossible to differentiate certain forms.

#### SUMMARY

A careful study of the salivary glands of normal and rabid dogs makes it apparent that they offer favorable conditions for the growth of certain protozoa, some of the developmental forms of which render differentiation between them and certain forms of Negri bodies impossible, or at least a very difficult task. It seems certain, however, that in many cases of rabies Negri bodies may be identified positively in the salivary glands.

## A PROTOZOAN PARASITE IN THE SALIVARY GLAND OF THE DOG

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An interesting parasite was found in the salivary glands of two old dogs that were used as controls in the work on rabies.<sup>1</sup> Nothing is known of the histories of these dogs, and the heads alone were received for examination. The animals were apparently well nourished. The tissues of the neck in both cases were edematous, and in one case the lymph glands were swollen and soft. The salivary glands had a peculiar yellowish color unlike the usual pinkish gray of these glands. In size and consistency they appeared normal.

On examination of the sections certain parts of the glands were found to be heavily infested with a peculiar protozoan parasite. In both cases the organisms were most abundant in the small anterior lobe of the submaxillary gland, and in one case they were also present in considerable numbers in the parotid, but in the latter location the forms were less varied.

The parasites are very numerous, many of them of large size and striking in appearance. All the cells of an acinus may contain parasites and a single cell may contain two parasites of different forms. A form which probably represents the vegetative or schizogenous stage consists of a deeply stained circular, central portion of variable size surrounded by a clear, radially arranged, unstained zone of varying width. These bodies are usually about 10 mikrons in diameter. The central portion is usually large, about 8 mikrons in diameter, and may be surrounded by a narrow clear zone, but often small, round bodies are seen budding off from the central body or lying free in the peripheral zone. Less frequently there is a small central body, 3-5 mikrons in diameter, with a radially arranged outer zone, sometimes without the small bodies, sometimes filled with the small bodies of quite uniform size and having a rather indefinite radial arrangement. The size of the small bodies seen in parasites with narrow peripheral zones varies considerably. While the majority of these forms are round, some are elongated and more or less irregular in

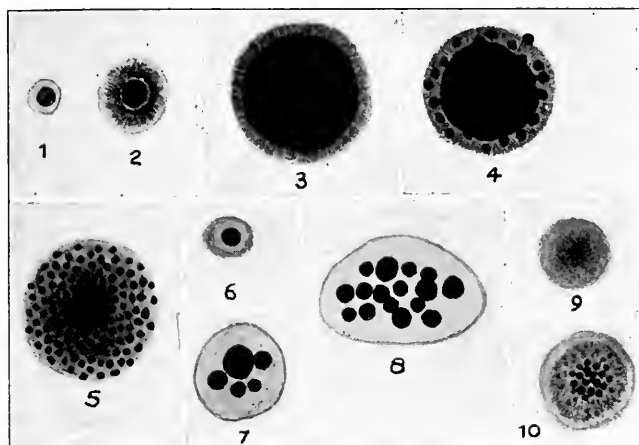
Received for publication May 19, 1921.

<sup>1</sup> Jour. Infect. Dis., 1921, 29, p. 291.

shape. The large parasites fill the cells they inhabit, but the wall of the cell remains, and one usually sees the nucleus of the cells flattened against the wall.

A much smaller and less deeply stained body, which may be an earlier form, is less often seen. In this body the central portion stains deepest, the color shading off toward the periphery without differentiation into central and outer parts.

Another form, which is present in about the same numbers as the first in one dog, and is almost the only form of the organism in the other dog, consists of a clear, lightly blue stained substance in which



Figs. 1, 2, 3, 4, 5.—Schizogenous forms. Figs. 6, 7, 8.—Sporogenous forms. Fig. 9.—Undifferentiated body. Fig. 10.—A body occasionally seen in duct epithelium.

are imbedded one or several round, deeply red stained bodies which differ considerably in size. This cyst-like form varies more in size than the vegetative, being 3 to 10 mikrons in diameter and sometimes of quite irregular shape. The small and medium sized organisms of this type are most numerous. Occasionally one sees a cyst filled with rather small bodies of uniform size. These forms I believe represent the sporogenous stage in the life cycle of the organism.

The vegetative stage of the parasite resembles somewhat that of the organisms found in the ducts of the salivary glands of guinea-pigs<sup>2</sup> and presumably represents a similar stage in the development of a

<sup>2</sup> Jour. Infect. Dis., 1920, 26, p. 347.

related parasite. In these dogs the forms of the organism just described have not been found in the duct epithellium, but occasional cells of the ducts of both the parotid and submaxillary glands of one case present an unusual appearance. Stained with eosin and methylene blue, these cells take a very deep red stain, have a swollen appearance and stand out distinctly among the less brightly stained normal cells. When examined carefully they are seen to contain many small red bodies, which are brought out distinctly by staining with iron hematoxylin. That these bodies are not secretion granules is evident from the following: They stain strongly with eosin while secretion granules do not; and with iron hematoxylin secretion granules stain more deeply, are smaller and more irregular in shape than these bodies, which are round and quite uniform in size, and the cells containing them desquamate. Rarely an epithelial cell of a duct is seen to contain a large round body with a deeply stained center and radiating peripheral zone, and I have been in doubt as to whether to regard it as a form of the parasite or not.

In the parotid glands of one of the dogs, the acinar cells contain considerable numbers of a smaller, more lightly stained, undifferentiated form. Similar forms occur in small numbers among the first described forms in the submaxillary gland, also in the glands of normal and rabid dogs, and consequently I am undecided whether they are forms of the parasite just described or an entirely different organism.

The lumen of the ducts, especially in infected portions of the submaxillary gland, contain many leukocytes and masses of deeply reddish stained, more or less homogeneous, material and in general have a very much distended appearance. Some of the parasites become detached from the acinar cells and are found lying free in the ducts, but are few in number. The ducts of uninvolved portion of the glands sometimes contain a few small oval ameba-like organisms similar to those so frequently found in the salivary glands of normal and rabid dogs. A peculiar feature of these glands is the comparatively slight reaction of the tissues to the extensive invasion of the parasite. In places in which the organisms are most numerous, there is little if any necrosis, few polymorphonuclear leukocytes in the tissues, and only a moderate amount of infiltration of mononuclear cells, most of which are plasma cells.

This organism apparently belongs among sporozoa since, according to Rivas,<sup>3</sup> some of the characteristic features of these protozoa are

<sup>3</sup> Human Parasitology, 1920, p. 146.



absence of pseudopodia, reproduction by spore formation, strictly parasitic nature requiring elaborated or predigested food for their nourishment, a simple structure consisting of cytoplasm and nucleus and a rather complicated life cycle. It seems probable that further classification would place them in class Telosporidia, group 2, Coccidia and family 4, Polysporocystidae.

## VIRULENT MICROCOCCUS CATARRHALIS IN INFLUENZA

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Several points of bacteriologic interest were brought out during the 1920 epidemic of influenza at the University of Wisconsin. During this epidemic, bacteriologic examination was made of the nasopharyngeal flora of 79 patients suffering from influenza, 15 patients in the hospital for other illnesses, a group of 26 medical students, a few of whom subsequently came down with influenza, and 9 patients with pulmonary tuberculosis. The medium used was for the most part heated blood agar (so-called "chocolate agar") made from a beef infusion basis, 5% defibrinated sheep blood and Witte's peptone (purchased before the war) and adjusted to a  $P_H$  of 7.4-7.6. In some instances unheated blood agar and Avery's oleate medium of the same H-ion concentration were also used. In addition to the plates from the nasopharyngeal swabbings, blood cultures and sputum examinations were made from some of the patients. In all instances the plates were streaked at the bedside, and the sputum examinations and animal inoculations were made without any material loss of time. An ordinary bent wire applicator was employed to obtain the material from the nasopharynx, and with reasonable care no contaminations were obtained by striking the swab against the tongue or other parts of the mouth.

A marked difference in the nasopharyngeal flora of the influenza patients was noted as compared with the results obtained by the same observers under essentially the same conditions during the more severe epidemic of 1918-19. The commonest organism in the throats of practically all the patients during the 1920 outbreak was a gram-negative coccus usually occurring in pairs. It was not infrequently present in almost pure culture on the chocolate-agar plates seeded with the nasopharyngeal swabbings. Organisms resembling *B. influenzae* were found only in small numbers in 10 instances. Members of the streptococcus-pneumococcus group were noticeable for their absence. They were rarely present, and only in relatively small numbers. In our

1918-19 epidemic, on the contrary, hemolytic streptococci and virulent pneumococci prevailed largely in the throats of the influenza patients, as well as among the students not suffering from this malady.

This gram-negative coccus isolated from the throats of influenza patients resembled *Micrococcus catarrhalis* in its morphology and cultural characters. On chocolate agar it grew readily in the form of slightly elevated, moist, translucent, nonpigmented colonies. On ordinary beef extract agar and on Löffler's blood serum the organisms grew less rapidly than on the richer mediums, but an abundant growth was obtained in from 36 to 48 hours. No pigment was produced even on the Löffler's blood serum. Growth in broth was slight, and the organism failed to ferment any of the ordinary carbohydrates, as determined by reaction to litmus solution.

When injected intravenously into rabbits in amounts of 1 to 2 slant cultures per animal, the organisms produced a profound toxemia. The animals were completely prostrated within 12 hours after the injection. They would lie on their sides showing marked depression, rapid shallow respiration, usually with some diarrhea. Death occurred in from 2 to 3 days. No lesions, except cloudy swelling of the viscera and occasionally subserous hemorrhages in the intestines, were observed. Strikingly enough, in no instance, in spite of diligent effort, were the organisms recovered from the heart blood or other organs of the body.

The same organism from a morphologic and cultural basis was also found abundantly in the noninfluenzal patients in the hospital. Unfortunately, the virulence of these organisms was determined in only one case but in that instance, one 24-hour culture injected intravenously into a rabbit produced no untoward symptoms.

The cultures from the tuberculosis patients were all taken on Feb. 11, after the epidemic at the university had subsided. The sanatorium in which these patients were is several miles out of the city, and influenza was not present in this institution nor had any of the patients been in contact with the outside world except through the nurses, physicians and occasional visitors. The cultures from these cases looked about normal for the time of year as to the incidence of streptococci and pneumococci, but there were more gram-negative cocci than one usually finds. These organisms were for the most part the pigmented species, but in two cases we found numbers of organisms that morphologically, culturally and on the basis of carbohydrate reac-

tions were of the catarrhalis type. In one other instance we found an organism which grew like *M. catarrhalis* but fermented glucose, the carbohydrate reaction of the gonococcus. None of these organisms produced any symptoms more serious than a slight rise in temperature following inoculation of several cultures into rabbits.

Agglutination tests with the gram-negative cocci isolated from influenza patients and also a strain of Pfeiffer's bacillus were made with serum from six of the convalescents, but in no instance were positive results obtained.

A study of the nasopharyngeal flora of a group of 26 medical students was carried on weekly throughout the winter and early spring months with another purpose in view. These examinations were made by M. S. Allen and D. G. Conover with the idea of obtaining information as to a possible relation of the nasopharyngeal flora with meteorologic conditions. Their findings need confirmation. It gives, however, additional information as to the incidence of *M. catarrhalis* or a similar organism among the general student population at this time. Ordinary blood-agar plates were used in this work, and a striking increase in the nonpigmented gram-negative cocci was observed during the epidemic period.

The epidemic began shortly after the return of the students from the Christmas vacation on Jan. 5, reached its peak with a total of 121 new cases Jan. 20, maintained approximately this level for 4 days, and then fell off, gradually disappearing altogether about Feb. 12 (Dr. J. S. Evans, personal communication). Large numbers of the catarrhalis type of organism were noticeable, especially during the peak of the wave, together with a marked falling off in the incidence of the members of the streptococcus-pneumococcus group. In fact, only 3 of the 26 showed any streptococcus-pneumococcus organisms at this time, whereas the average during the winter months showed a positive finding of these organisms in 12 of the 26. None of the organisms from this group were tested for virulence.

Obviously the results here reported suggest no etiologic relationship between the gram-negative coccus isolated and epidemic influenza. During this 1920 epidemic, however, this organism, which corresponds to the usual description of *M. catarrhalis*, was markedly enhanced in virulence by the growth under the conditions prevailing in the body of influenza patients. Similar organisms isolated from noninfluenzal cases at about the same time did not show this increased virulence. Whereas

ordinarily many cultures of this organism may be injected into rabbits without causing more than a slight transitory rise in temperature, in this instance doses as small as a single culture produced a fatal termination in 2 or 3 days. Apparently this is another rather striking instance of the capacity of the influenzal infection to increase the virulence of other associated organisms. This epidemic, as has been suggested by others, was less serious than the epidemic of a year before, presumably because of the low incidence of organisms that have the capacity of acquiring a high virulence for human beings, namely, streptococci and pneumococci such as were so abundantly present during the winter of 1918-19.

In view of these findings, it would seem as though gram-negative cocci other than the meningococcus and the gonococcus deserve more attention than they receive. Some years we have not infrequently found gram-negative cocci as the predominating organisms in throat cultures or in conjunctivitis or middle ear infections. Then for several years they may occur only rarely, and those present are usually the pigmented species. Should not all throat cultures, including those from cases of suspected diphtheria, be stained by Gram's method as a routine procedure? We believe that important additional information as to the rôle of *M. catarrhalis* and similar organisms would thus be obtained.

# ON THE CLAIM THAT SOME TYPHOID-PARATYPHOID STRAINS SURVIVE THE MILK PASTEURIZATION

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The statement of Schorer and Rosenau<sup>1</sup> that the thermal death points of the pathogenic organisms that may render milk dangerous have been determined with precision in many laboratories and that an actual exposure to 60 C. for 20 minutes would suffice to kill such nonspore-bearing organisms as *B. diphtheriae*, *B. tuberculosis* and *B. typhosus* voiced the general conclusion of all who had worked on this subject. Although the practical application of these facts to the pasteurization of large volumes of milk was another problem, exposure to this temperature, for the time given, had been uniformly considered as adequate, and this aspect of the subject was looked on as closed.

Recently, however, Twiss<sup>2</sup> has reopened the subject and used bacteria of the typhoid-paratyphoid group as test organisms. Her contention is that, in actuality, the temperatures of pasteurization are inadequate; that the method of using small samples of the heated milk for the determination of the death of the bacterium present does not exclude the possibility of the survival of a few organisms. She asserts that if the whole test sample (100 c c) be incubated it will not infrequently be found that a few bacilli have survived exposure to 60 and even to 65 C. for 30 minutes.

The practical importance of the question immediately presented itself, and we began testing a series of cultures from different sources. Twenty-seven typhoid cultures recently isolated from carriers, 7 paratyphoid A cultures, 12 paratyphoid B. cultures and 4 enteritidis cultures were subjected to experiments, using the ordinary temperature of pasteurization.

Milk was sterilized in the autoclave, cooled and tested for sterility. It was then distributed in measured quantities in sterile containers. The containers were placed in wire baskets on a perforated tray in a water bath, and the temperature of the bath and the milk

Received for publication May 25, 1921.

<sup>1</sup> Jour. Med. Research, 1912, 26, p. 127.

<sup>2</sup> Jour. Infect. Dis., 1920, 26, p. 165.

## DO TYPHOID-PARATYPHOID STRAINS SURVIVE PASTEURIZATION 311

raised to 60 C. After this temperature was reached, suspensions of the cultures in salt solution were poured into the milk. Rubber stoppers were inserted, the bottles shaken vigorously and quickly returned to the water bath in which they were completely submerged. The temperature of 60 was held during the pasteurization period of 15 minutes. Rapid cooling was accomplished by plunging the samples into a bucket of cold water held under an open faucet. This method of pasteurization excluded the preliminary heating of the bacteria while the temperature of the milk was being raised. In every one of these tests we used a volume of 100 c c of sterilized milk infected with the cultural growth from two 24-hour agar slants suspended in salt solution.

TABLE 1  
SUMMARY OF PASTEURIZATION EXPERIMENTS WITH TYPHOID, PARATYPHOID AND ENTERITIDIS STRAINS

Cultures Tested		Volume in C c of Milk Used in Tests	Infected With Growth From	Temperature of Milk When Infected	Minutes to Reach 60 C.	Minutes That Tests Were Heated at 60 C.	Results of Tests
B. typhosus	27 strains	100	2 agar slants	60 C.	—	15	No growth
B. paratyphosus A	7 strains	100	2 agar slants	60 C.	—	15	No growth
B. paratyphosus B	12 strains	100	2 agar slants	60 C.	—	15	No growth
B. enteritidis	4 strains	100	2 agar slants	60 C.	—	15	No growth
B. paratyphosus B	No. J* 202	100	2 agar slants	60 C.	—	15	No growth
B. paratyphosus B	No. J* 245	100	2 agar slants	60 C.	—	15	No growth
B. paratyphosus B	No. J* 252	100	2 agar slants	60 C.	—	15	No growth
B. paratyphosus B	No. J 202	400	10 agar slants	60 C.	—	10	No growth
B. paratyphosus B	No. J 202	400	10 agar slants	60 C.	—	20	No growth
B. paratyphosus B	No. J 245	400	10 agar slants	60 C.	—	10	No growth
B. paratyphosus B	No. J 245	400	10 agar slants	60 C.	—	20	No growth
B. paratyphosus B	No. J 252	400	10 agar slants	60 C.	—	10	No growth
B. paratyphosus B	No. J 252	400	10 agar slants	60 C.	—	20	No growth
B. paratyphosus B	No. J 202	300	1 agar slant	25 C.	17	10	No growth
B. paratyphosus B	No. J 202	300	1 agar slant	25 C.	17	20	No growth
B. paratyphosus B	No. J 245	300	1 agar slant	25 C.	18	10	No growth
B. paratyphosus B	No. J 245	300	1 agar slant	25 C.	18	20	No growth
B. paratyphosus B	No. J 252	300	1 agar slant	25 C.	10	10	No growth
B. paratyphosus B	No. J 252	300	1 agar slant	25 C.	10	20	No growth

\* Received from Dr. Jordan.

The inoculated milk samples were then incubated for 48 hours at 37 C. to allow multiplication of any organisms which survived. After incubation, samples in duplicate, 0.5 c c and 0.1 c c, were plated in agar and 2 broth tubes were each inoculated with 0.1 c c. Russell's medium was also inoculated from the infected milk, as well as from the inoculated broth tubes.

None of the micro-organisms tested survived a pasteurization period thus limited closely to 15 minutes at 60 C.

These experiments confirmed our previous experiences, as well as those of others. Whether or not there might be more resistant members of the typhoid-paratyphoid group could not be excluded as a possibility, although this seemed improbable. To exclude the possibility that Twiss was working with unusually heat resistant strains, we obtained some of the strains which in her experiments seemed to survive the usually accepted pasteurization period.

The resistance of these strains was tested in several ways. One set of experiments with 100 c c of milk was carried out as described, and no growth was obtained. Then, larger volumes were inoculated very heavily by using for each bottle the growth from 10 agar slants. Whether the milk was inoculated before or after reaching 60 C., the results were the same. No growth was obtained after 20 minutes or even after 10 minutes' heating at 60 C.

Repetitions of these tests, not recorded here, have given the same negative results.

It would seem that the apparent heat resistance of the strains reported by Twiss was due to the test method employed, namely, the use of cotton plugged flasks submerged to twice the depth of the milk. The possibilities of discordant results from this open method have been pointed out by Smith<sup>3</sup> and others.

#### CONCLUSIONS

There is no evidence that bacilli of the typhoid-paratyphoid group, even in small numbers, will survive heating to 60 C. for 20 minutes. To insure actual heating of all the sample and to exclude other sources of error in laboratory pasteurization tests, the sample container should be completely submerged in the bath.

<sup>3</sup> Jour. Exper. Med., 1899, 4, p. 233.



# THE BACTERIOLOGY OF THE BLOOD OF DOGS WITH ECK FISTULA

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There is an impression, supported by considerable evidence, that organisms from the bowel often enter the portal stream and in the liver are effectively disposed of. In order to test this point it was thought that Eck fistula dogs might furnish proper conditions; at least, it would be of interest to note whether or not bacteria appeared in the general circulation or possibly caused occasional infections in the lungs or elsewhere in such animals.

An Eck fistula is an artificial communication between the portal vein and the vena cava with the portal vein ligated above the anastomosis just at the hilus of the liver. This operation destroys the portal circulation so far as the liver is concerned, in that now the flow of blood from the abdominal viscera (except the kidneys and the suprarenal glands) is returned to the general circulation without passing through the capillaries of the liver; in other words, the liver is shunted out of the portal circulation and receives its sole blood supply by way of the hepatic artery, which carries about one-fifth of the quantity of blood that is carried by the portal vein.

The anatomic situation of the liver naturally suggested to investigators a protective function. Under normal condition all the products of intestinal digestion and other absorbable substances (except the greater part of the fats) must first pass through the liver capillaries before reaching the tissues in general, while under the conditions imposed by an Eck fistula they reach the tissues first. It has been amply proved that the liver exercises a protective function against certain poisons absorbed from the alimentary tract, especially against certain of the products of protein digestion, such as ammonia. In fact, dogs with Eck fistulas will not tolerate a heavy meat diet.

To prove whether or not bacteria actually pass the bowel wall into the circulation and, if so, what influence the liver has on them, we instituted the following experiments on dogs with Eck fistula.

June 25, an Eck fistula was made in a dog weighing 19,000 gm. Recovery was prompt without infection; some emaciation followed. On July 9, 12, 13 and 16 from 5 to 10 c.c. of blood were drawn from the femoral vein under sterile precautions and introduced into 3 or 4 culture tubes of broth. Of the tubes inoculated, only one taken on the first day yielded a slight growth of a nonhemolytic small streptococcus.

An identical experiment was made with a second dog with Eck fistula operated on July 4. The cultures were made on July 12, 13 and 16. The various tubes were inoculated with the blood as in the first experiment, and all remained sterile indefinitely. These experiments were more or less in the nature of preliminary tests to determine whether or not bacteria in dogs with Eck fistula passed through the bowel wall and remained in the circulation for any length of time. The results we regard as negative. In only one of the tubes did we obtain a slight growth of a small coccus which we believe to be a contaminator.

In the next experiment the conditions were altered somewhat. On July 10, an Eck fistula was made in 2 normal dogs by one of us (Mathews). No untoward results followed the operation. Seventeen days later when healing was complete blood was drawn, with proper precautions, from the femoral vein. From each dog about 5 c.c. of the fresh blood was introduced into 4 large tubes containing 50 c.c. of ascites broth; 2 were placed under anaerobic and 2 under aerobic conditions. The tubes were kept under observation for a period of 2 weeks and in none did a growth occur. The dogs in this experiment were bled 2 hours after a rather heavy meal of meat, with the idea that possibly the absorption might favor the passage of bacteria through the bowel wall. Evidently this is not the case.

Again on Aug. 2, 23 days following the operation the 2 dogs were bled from the right side of the heart by needle puncture. Some of the blood thus obtained would come directly from the portal circulation without passage through either the liver or lung capillaries.

This time the dogs were bled 4 hours after eating heavily of meat. Cultures were made in ascites broth as before and observed from time to time. No organisms grew in any of the tubes either under aerobic or anaerobic conditions. The results of both of these experiments, then, were negative for the circulating blood of these animals under the condition stated.

Blood from these dogs was then examined bacteriologically after the introduction of cultures of bacteria into the stomach. The bacteria were introduced by means of the stomach tube and 100 c.c. each of the 48-hour cultures of *B. pyocyaneus* and *B. subtilis* were so given to both dogs on a fasting stomach. After 4½ hours, from the heart of each dog 10 c.c. of blood were removed by needle puncture and introduced into 4 broth tubes in diminishing quantities. Neither *B. subtilis* nor *B. pyocyaneus* was obtained from any of the tubes following incubation. From dog 1 a small coccus grew slowly in 2 tubes which was not identified. From dog 2 a larger coccus grew profusely in one tube in 24 hours. We felt justified in regarding these as contaminations.

A week later the same experiments were repeated and 10 c.c. of blood were taken from the heart for culture at the end of 1 hour, 2 hours and 5 hours. Four broth tubes were inoculated from each sample of blood and 2 were placed under aerobic and 2 under anaerobic conditions. These cultures all remained without growth of *B. pyocyaneus* and *B. subtilis*. In 2 instances a small coccus appeared in few numbers, similar to the one obtained previously and which was regarded as a skin saprophyte.

About one week following this experiment the dogs were killed and examined. The Eck fistulae were in perfect condition. No important change was noted in the organs.

The next experiment was designed to compare the length of time bacteria artificially introduced into the circulation remain in dogs with Eck fistula and in normal dogs. In dog 1, several days following the last experiment 8 cc of a 36-hour broth culture of a staphylococcus albus was injected intravenously. As a control, at the same time a normal dog of approximately the same weight was injected with a similar amount. Blood cultures were made at short intervals from both dogs. The results are presented in table 1.

Between 2 and 3 hours after the injection both dogs became sick and vomited. After 24 hours they appeared quite normal. It will be noted that the staphylococci in 24 hours had disappeared from the blood, and at the end of 5 hours had somewhat diminished. It is possible that had many more cultures been made, slight differences might have been detected. It is apparent, however, that no appreciable differences appeared in the elimination of bacteria by the 2 dogs. A second similar experiment was made. Accidental contamination of some of the plates occurred, but so far as the results could be determined, they correspond with those of the first experiment.

TABLE 1  
LENGTH OF TIME BACTERIA, ARTIFICIALLY INTRODUCED, REMAINS IN NORMAL DOGS AND IN DOGS WITH ECK FISTULA

Time After Injection	Growth from Normal Dog	Growth from Dog with Eck Fistula
5 min.	+++	+++
10 min.	+++	+++
15 min.	+++	+++
30 min.	+++	+++
1 hour	+++	+++
2 hours	+++	+++
3 hours	++	++
5 hours	++	+
24 hours	0	0

In this connection it may be mentioned that dogs with Eck fistula do not appear to be especially susceptible to infection. During a period of 10 years, one of us (Mathews) has had under observation about 100 dogs with Eck fistula, living from a few weeks to 3 years and noticed no more tendency to infection than in normal dogs. One thing noted, however, was that about 10% of these dogs failed to maintain a normal state of nutrition regardless of food or hygienic care. They suffered from diarrhea, and the stools were fatty and similar to the bowel discharges following ligation of the common bile duct. These dogs died in an extreme state of inanition after 2 or 3 months. Ulcers of the duodenum were observed in 5, and perforation was the immediate cause of death in 2. The diarrhea and fatty stools were probably due to a suppression of the formation of bile, which has been observed by Voegtlin and others in dogs with Eck fistula.

The obvious relation of the entrance of bacteria to the problem of so-called focal infection need only be mentioned. In spite of the negative character of our data, the suggestion is here offered that in a given case in seeking the source of the infection we should not overlook the possibility that the bacteria entered the body along a route long since closed with no evidence now of its past existence. Or they may have passed through the intact membrane without causing any local alterations, as tubercle bacilli may do for example, in passing through the intestinal wall.

Our results in general are in accord with observations made by many on the relatively rapid disappearance of bacteria when introduced into the circulation. Bull<sup>1</sup> has called attention to the intravascular agglutination of bacteria and their subsequent accumulation in the lungs, liver, and spleen. The endothelium, especially in certain organs, is most active in the destruction of the organisms.

Bartlett and Osaka,<sup>2</sup> investigating the fate of micrococcus aureus in normal dogs, came to the conclusion that they are rapidly stored up in large numbers in the lungs following injection into the left ventricle. A little later they disappear in the lung and appear in considerable numbers in the spleen and liver but again disappear from here in from 48 to 72 hours. In blood, bone marrow, lymph nodes, muscles, etc., they are found in small numbers or not at all. Their method of detection was by means of sections, and they reported no cultural results.

In dogs with Eck fistula in all probability the bacteria in the circulation, however they may have entered, are disposed of by the various mechanisms referred to. It seems that no appreciable differences occur in this respect between normal dogs and those with Eck fistula. Though the amount of blood that flows through the liver is markedly diminished in the latter, apparently this does not appreciably alter the general rate of destruction of the bacteria, since so many cells and organs other than the liver are concerned in this process.

#### SUMMARY

Experiments do not indicate that in dogs with Eck fistula bacteria in any appreciable numbers appear in the circulation.

Dogs with Eck fistula are no more susceptible to infection of the lungs or of other organs than are normal dogs.

Bacteria disappear from the circulation as rapidly in dogs with Eck fistula as in normal dogs.

<sup>1</sup> Jour. Exper. Med., 1915, 22, p. 475.

<sup>2</sup> Jour. of Med. Res., 1916, 35, p. 465.

## A CONSTRICTED TUBE WITH MECHANICAL SEAL FOR ANAEROBIC FERMENTATION TESTS

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No attempt is made to chronicle the various devices invented to meet the peculiar requirement of reduced oxygen tension in anaerobic fermentation tests. I was more interested in a method which would reduce that difficulty to a minimum and at the same time contribute to a proper understanding of the test itself. After trying mercury as a seal in the Smith fermentation tube, which fails on account of its germicidal action, and sand, which works but is troublesome, it was decided to combine the principle of the constricted tube (Hall,<sup>1</sup> 1915) with that of the fermentation tube so as to use a mechanical seal.

Since the paper in which the constricted tube was first described did not have a wide circulation, an illustration which appeared therein is reproduced here (Fig. 1) in order to show several types of seal that may be used. Glass, imitation agate, marbles are best, but we have also used a porcelain biconvex disk manufactured by the Star Porcelain Co. of Trenton, N. J., for this purpose, as well as circular cover slips. The two latter have the advantage of permitting the passage of a pipet into the lower portion of the tube, but the seal is less perfect than that of the marble. The mechanical seal principle has been used in flasks also, as shown in fig. 2.

The use of the mechanical seal principle in a fermentation tube is represented in fig. 3. The first lot of tubes was made for the writer at the University of Chicago in 1920. These first tubes were designed with the anaerobic arm of the tube closed, but difficulty in cleaning and a desire to obtain culture material from the closed branch without admixture with that portion of medium exposed to the air above the seal, suggested that the anaerobic arm be closed with a rubber stopper as indicated. The dimensions used are: diameter of open branch 25 mm., diameter of closed branch 9 mm., length 145 mm. Several lots recently made by Mr. W. J. Cummings, glass-blower in the Department of Chemistry at the University of California, according to these

Received for publication May 29, 1921.

<sup>1</sup> University of California Publications in Pathology, 1915, 2, p. 147.

specifications have cost \$30 per 100. The tubes have proved to be somewhat fragile, especially if care is not exercised when inserting the rubber stopper to hold the tube by the small branch, but broken tubes are readily repaired by a competent glass-blower at slight expense. Some difficulty was encountered in securing rubber stoppers to fit the closed branch, as a size smaller than those regularly listed was required;

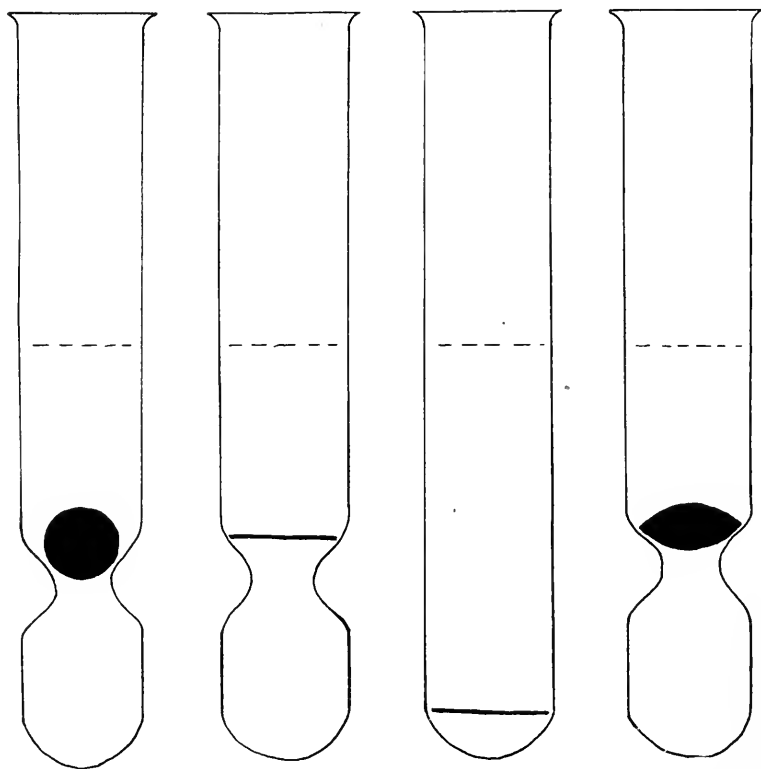


Fig. 1.—Mechanical seals for anaerobic cultures; (a) marble seal in constricted tube; (b) cover slip seal in constricted tube; (c) cover slip in plain tube; (d) biconvex disk in constricted tube. The dotted line represents the height of liquid medium in each tube.

we are now using a good grade of red gum stopper especially manufactured for the Cutter Biological Laboratories of Berkeley, and kindly supplied by them to us. The glass imitation agate marbles used vary in diameter from 15 to 20 mm. The tubes are best plugged for use with a cotton plug covered with surgeon's gauze.

The use of this device is by no means limited to fermentation reactions. It provides a valuable means of supplying culture material from the closed branch for motility tests with a minimum of exposure to air. Such tubes also may serve, with or without the seal, every purpose that the original Smith fermentation tubes serve. Were it not

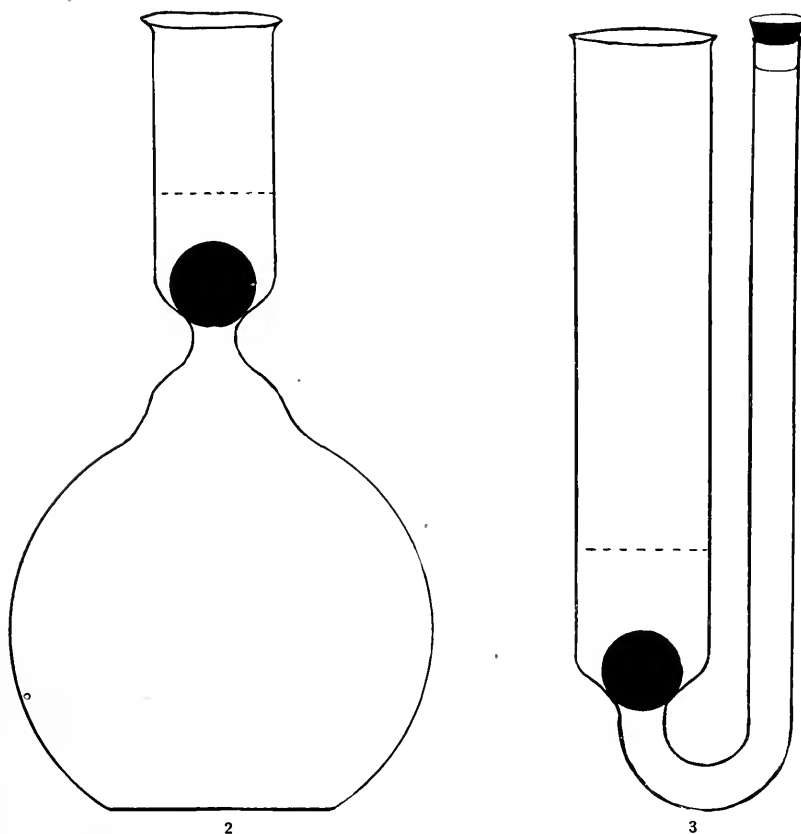


Fig. 2.—Constricted neck flask with marble seal. The dotted line represents the height of liquid medium in the flask.

Fig. 3.—Fermentation tube with marble seal. The dotted line represents the height of liquid medium in the tube.

for the greater expense, the constricted fermentation tube might serve every purpose that the ordinary constricted tube serves, aerobic and anaerobic sterility tests, virulence tests, preparation of immunizing and agglutinating antigens, and all the varied purposes for which a small volume of broth culture of any obligate anaerobe may be desired.

But the principal reason for the invention of this fermentation tube was that an adequate study might be made of the problem of utilizing the fermentation tests of obligate anaerobes in a differential and taxonomic sense, having in mind the fact that peculiar obstacles are encountered in the proper interpretation of the usual criteria of fermentation when applied to these organisms.



## CRITERIA IN ANAEROBIC FERMENTATION TESTS

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The most fundamental premise in the differentiation and identification of micro-organisms through determination of their metabolic functions rests in the unquestioned purity of the culture under study; a mixture of species gives, as a rule, a result that represents the sum of the properties possessed by the individual strains represented. On account of the confusions growing out of fermentation and other cultural tests conducted with impure cultures, little headway was made previous to the war in the taxonomy of the obligate anaerobes.

Impurity of culture need no longer be considered an insurmountable difficulty in the taxonomy of the anaerobes, for, while an almost meticulous anxiety must be exercised in order to avoid contaminations, both deep and surface culture methods exist which suffice for isolation if repeatedly and conscientiously applied. Except in rare instances the purification problem is a solved problem.<sup>1</sup>

Assuming the problem of purity to be settled, one is justified, in view of the great utility of the fermentation reactions in aerobic bacteriology, in inquiring to what extent they may be applied in the differentiation and identification of obligate anaerobes. There has been until recently a widespread impression that the usual (i. e., aerobic) criteria of fermentation, namely, acid and gas production, encounter serious limitations in the interpretation of fermentation tests of anaerobes. It is the purpose of this paper to formulate these limitations in the belief that the taxonomic utility of the fermentation reactions is no less for the obligate anaerobes than for the aerobes, providing certain conditions are met. The constricted fermentation tube with mechanical seal<sup>2</sup> has been of considerable aid in this study.

### WHAT IS THE TRUE CRITERION OF ANAEROBIC FERMENTATION?

The writer's attention was first drawn to the difficulties in the proper interpretation of anaerobic fermentation tests in 1916. Certain cultures in our possession at that time could be divided into two metabolic

Received for publication June 6, 1921.

<sup>1</sup> Hall: *Jour. Infect. Dis.*, 1920, 27, p. 576

<sup>2</sup> Hall: *Ibid.*, 1921, 29, p. 314.

groups; in one there was a marked putrefactive tendency as shown in the blackening and digestion of a brain medium; in the other there was no such tendency. This distinction was equally marked in the action of these organisms on milk in the constricted tube.

In the putrefactive group growth in milk was at first slow, often not evident at all until after 36-48 hours, when a soft solid curd with little or no gas was formed. After 72 hours liquefaction had usually begun, accompanied by some gas production as the coagulated casein digested. Such cultures were always foul in odor.

In the nonputrefactive group several types of change were noted, such as rapid coagulation of casein with vigorous, "stormy" gas production, slow coagulation with moderate gas formation, gas formation without coagulation or no visible change. In such cultures there was never liquefaction or digestion of the casein, and the odor was not foul.

It was surprising to find that the "titratable acidity," using phenolphthalein as an indicator, was frequently higher in milk cultures of the putrefactive type than in milk cultures of the nonputrefactive group. But with our growing appreciation of the importance and significance of the  $H^+$  ion concentration, and our belief that lactose could not be fermented in milk without inhibiting putrefaction, it was not surprising to find that the reaction of the putrefactive cultures had actually changed slightly in the direction of alkalinity without, however, passing the changing point of phenolphthalein. These results have been twice repeated recently with a representative group of carefully purified and identified cultures, as shown in table 1.

Fresh pasteurized cow's milk was sterilized in the new constricted fermentation tube with marble seal <sup>2</sup> by the intermittent method. Daily observations on the macroscopic changes were made for one week. Gas production was estimated with Frost's gasometer. Aerobic contamination was excluded by subculturing on the surface of plain agar plates, all of which remained barren on incubation.

The direction of reaction change was determined by withdrawing three 0.5 c c portions of culture fluid from the closed branch of the tube after removing the rubber stopper thereof, placing them in the depressions of a white porcelain test plate and adding to one, 2 drops of 0.04% aqueous solution of brom-thymol-blue, to another, 2 drops of 0.02% alcoholic solution of methyl-red, and to the third, 2 drops of 0.04% aqueous solution of brom-phenol-blue.

The titer was determined with two indicators, namely, phenolphthalein and brom-thymol-blue.

Of the first, 1 c.c. of a 0.5% solution in 50% alcohol was added to 5 c.c. of culture fluid diluted with 25 c.c. of neutral distilled water. This mixture was brought barely to boiling and titrated while hot to the first permanent tint of pink with N/20 NaOH.

Of the second indicator, 1 c.c. of 0.04% aqueous solution was added to 5 c.c. of culture fluid likewise diluted with 25 c.c. of neutral distilled water but titrated cold against N/20 NaOH to the color of 30 c.c. of a standard buffer solution ( $P_H=7.0$ ) with an equal amount of dye in a white evaporating dish.

TABLE 1  
ACTION OF CERTAIN OBLIGATE ANAEROBES IN MILK IN THE CONSTRICTED FERMENTATION TUBE

Culture No.	Culture Name	Visible Change	Total Gas, %	Titer to Phenolphthalein	Titer to Brom-thymol-blue	Terminal Reactions	Reaction Change
Control	Uninoculated	None	0	+2.8	+1.8	Alkaline to methyl-red; slightly acid to brom-thymol-blue	None
1	<i>B. tetani</i>	None	0	+2.9	+1.6	Alkaline to methyl-red; acid to brom-thymol-blue	Sl. alk.
2	<i>B. welchii</i>	Stormy fermentation	100	+8.4	.....	Alkaline to brom-phenol-blue; acid to methyl-red	Acid
4	<i>Vibrio septique</i>	Slow coagulation only	0	+3.7	2.1	Alkaline to brom-phenol-blue; acid (?) to methyl-red	Acid
8A	<i>B. botulinus</i>	Putrefactive	8	+10.8	2.3	Alkaline to methyl-red; nearly neutral to brom-thymol-blue	Alk.
10	<i>B. sporogenes</i>	Putrefactive	5	+8.0	3.5	Alkaline to methyl-red; nearly neutral to brom-thymol-blue	Alk.
50	<i>B. bifermentans</i>	Putrefactive	8	+8.7	2.5	Alkaline to methyl-red; nearly neutral to brom-thymol-blue	Alk.

On account of the opacity of the undigested casein in certain cultures and the marked color in those cultures of which the casein was digested, it was impossible to apply colorimetric methods to the exact determination of the terminal  $H^+$  ion concentrations; but by comparing the color in the test plate of equivalent amounts of culture fluid containing equal quantities of brom-thymol-blue, the direction of the reaction change could be determined as noted.

The reaction change of the putrefactive cultures, *B. botulinus*, *B. sporogenes*, and *B. bifermentans* was distinctly in the direction of alkalinity as contrasted with the uninoculated control, that of the non-fermentative, slightly putrefactive *B. tetani* slightly so. It is

notable that in none of these was the reaction distinctly alkaline to the true neutral point, i. e.,  $P_H=7$ . Yet while the markedly putrefactive cultures (8A, 10 and 50) indicated a higher OH-concentration than *B. tetani*, they also required more alkali to bring them to the true neutral point, as shown in the titration values. The latter must therefore be interpreted as indicative of a great increase in buffer capacity through proteolysis. Foster and Randall<sup>3</sup> have confirmed Robertson's explanation of this phenomenon which considers that the  $-\text{COHN}-$  groups of the protein molecule become available for neutralization of acids and bases during hydrolysis.

*B. welchii* and *Vibrio septique*, especially the former, gave distinct changes in the direction of acidity.

Of the nonputrefactive organisms, only *B. welchii* produced a titratable acidity comparable with those of the putrefactive organisms. The unreliability of using titratable acidity as a criterion of the direction of reaction changes is strikingly displayed. Even the use of bromthymol-blue may lead one astray in the case of milk because, while certain putrefactive organisms may change the reaction in the direction of alkalinity, this change may not even reach the true neutral point. The only proper point of reference therefore is the reaction of the uninoculated control.

Gas was produced in slight amount by all the putrefactive cultures and abundantly by *B. welchii*.

The cultures mentioned in table 1 must be considered not only from the standpoint of their obvious action on casein, but also from the standpoint of their known action on carbohydrates. Three groups are represented. *B. tetani* is to be regarded as not directly fermenting any carbohydrate.<sup>4</sup> *B. Welchii*,<sup>5</sup> *Vibrio septique*, and *B. Chauvoei*<sup>6</sup> ferment both monosaccharides and the disaccharide lactose, while the putrefactive anaerobes, *B. botulinus*, *B. sporogenes* and *B. bifermentans* ferment only monosaccharides.<sup>7</sup>

No one will question the fermentation of milk sugar by *B. welchii*, *Vibrio septique* and *B. Chauvoei*. But Wolfe and Harris<sup>8</sup> have shown that the lactose of milk is consumed by certain putrefactive

<sup>3</sup> Foster: Jour. Bacteriol., 1921, 6, p. 143.

<sup>4</sup> Adamson: Jour. Path. & Bacteriol., 1920, 6, p. 143.

<sup>5</sup> Simonds: Monograph No. 5, Rockefeller Inst. for Med. Research, 1915. Jablons: Jour. Lab. and Clin. Med., 1920, 5, p. 374. Esty: Jour. Bact., 1920, 5, p. 375.

<sup>6</sup> Robertson: Brit. Med. Jour., 1918, 1, p. 583.

<sup>7</sup> Weinberg and Séquin: La Gangrène gazeuse, 1918.

<sup>8</sup> Jour. Path. & Bacteriol., 1917, 21, p. 386.

anaerobes, notably *B. sporogenes*, and this view is further supported by the failure of our putrefactive organisms to carry the alkalinity past the neutral point. How can this apparent fermentation of lactose in milk with acid and gas production be harmonized with the fact that in deep litmus-lactose agar one produces some gas, it is true, but only an alkaline reaction with these putrefactive anaerobes? The following explanation is offered. It may be presumed that the initial curd formation is due to acid formed from the slight trace of glucose present in milk. When this is exhausted proteolysis begins and sufficient ammonia is generated to begin the hydrolysis of lactose to glucose. From that time forward in the cultures fermentation and proteolysis go on side by side in the culture maintaining for days a balance that finally results in the almost complete destruction of both the high protein content and the high lactose content of the milk.

It was at this point that a critical survey of the problem of anaerobic fermentation was begun. For purposes of discussion gas and acid as criteria of fermentation are considered separately although in the experiments the possible correlation of these phenomena was kept constantly in mind.

#### GAS AS A CRITERION OF ANAEROBIC FERMENTATION

With aerobic bacteria gas formation is universally regarded as one evidence of fermentation.

The use of gas production as a criterion in fermentation tests of obligate anaerobes was suggested by Theobald Smith<sup>9</sup> in 1890. Smith proposed the use of mercury as an air seal in the fermentation tube, but a recent letter from Professor Smith states that he never tried it, and the writer in collaboration with Miss Jeanette Gay of the University of California has shown that mercury cannot be used as a seal on account of its inhibition of growth. Smith<sup>10, 11</sup> later overcame the initial failure of anaerobes to grow in the fermentation tube by the narrowing of the neck of the tube and by the use of sterile tissues in the medium, and with Brown and Walker<sup>12</sup> studied the gas production of several anaerobic cultures. The production of some gas in mediums presumably freed from sugar by *Bact. coli* to which tissues were subsequently added, was recognized as an obstacle in the interpretation of the tests. The omission of tissues was therefore advised. Randall and Hall<sup>13</sup> have recently shown the inadequacy of *Bact. coli* in the preparation of sugar-free mediums for fermentation tests of anaerobes.

<sup>9</sup> Centralbl. f. Bakteriöl., 1890, 7, p. 502.

<sup>10</sup> The Fermentation Tube, Wilder Quarter Century Book, Ithaca, N. Y., Comstock Pub. Co., 1893.

<sup>11</sup> Centr. f. Bakt., 1895, 18, p. 1.

<sup>12</sup> Jour. Med. Research, 1905, 14, p. 193.

<sup>13</sup> J. Infect. Dis., 1921, 29, p. 314.

Meyer,<sup>14</sup> Robertson,<sup>15</sup> and Esty<sup>5</sup> and others have at least tacitly regarded gas along with acid as a criterion of sugar splitting in the fermentation tube. Epstein<sup>16</sup> invented a special form of apparatus for the study of anaerobic gas, and Beattie<sup>17</sup> used the gas formation under petrolatum in minute tubes as an economical method of determining the fermentation reactions of anaerobes.

Henry<sup>18</sup> clearly recognized the difficulties in the problem. Studying the reactions in a neutral casein digest tubed in a Durham fermentation tube with an oblique vial similar to the writer's modification,<sup>19</sup> he concluded that while both gas and acid may serve as criteria of anaerobic fermentation, gas is of doubtful value unless produced abundantly during the first 24 hours of incubation.

Some writers have proposed to regard carbon dioxide as the proper criterion of fermentation. Smith, Brown and Walker<sup>12</sup> particularly emphasized the  $\frac{\text{CO}_2}{\text{Total gas}}$  ratio. However, Keyes,<sup>20</sup> and Keyes and Gillespie<sup>21</sup> showed that the quantitative significance of gas ratios obtained from the ordinary fermentation tube is doubtful. Notwithstanding, Todd<sup>22</sup> and Simonds<sup>6</sup> have since gone to considerable pains to record such values for certain anaerobes. Apparatus and methods to obviate the well-known sources of error in the gas ratios so determined have been most highly developed by Wolf, Harris and Telfer.<sup>23</sup>

It occurred to the writer that the marble seal in the constricted fermentation tube might suppress outward diffusion of carbon dioxide sufficiently to yield gas ratios comparable in accuracy to those obtained by more elaborate methods. Mr. Noah Fox collaborated at the University of Chicago in an attempt to correlate the ratios as determined for glucose broth in the constricted tube with marble seal and in sealed flasks.

In this work, meat infusion 1% glucose broth,  $P_H=7$ , was filled into the tubes and also into small Erlenmeyer flasks of about 200 c c capacity. The flasks were filled about half full, and the flasks were stoppered with rubber stoppers perforated to receive glass stopcocks. With some caution these were sterilized and inoculated in duplicate while yet warm with cultures under study. They were immediately evacuated with an oil vacuum pump and incubated at 37 C. Parallel cultures were made in constricted fermentation tubes.

Following various periods of incubation the flasks were connected with a Hempel gas apparatus, and since no mercury pump was avail-

<sup>14</sup> Jour. of Infect. Dis., 1915, 17, p. 458.

<sup>15</sup> Jour. Path. & Bacteriol., 1916, 20, p. 327.

<sup>16</sup> Centralbl. f. Bakteriol., 1898, 24, p. 266.

<sup>17</sup> Brit. Med. Jour., 1916, 1, p. 756.

<sup>18</sup> Jour. Path. & Bacteriol., 1917, 21, p. 344.

<sup>19</sup> Hall: Amer. Jour. Public Health, 1914, 4, p. 1173.

<sup>20</sup> Jour. Med. Research, 1909, 21, p. 69.

<sup>21</sup> Jour. Biol. Chem., 1912-13, 13, pp. 291 and 305.

<sup>22</sup> Jour. Infect. Dis., 1917, 5, p. 20.

<sup>23</sup> Jour. Path. & Bacteriol., 1916, 21, p. 386; 1918, 22, pp. 1 and 115; 1919, 23, p. 30; Biochem. Jour., 1917, 21, pp. 197 and 213.

able, the dissolved gas contained in the liquid, after the initial release of pressure, was driven off by boiling gently. This procedure was recognized as a possible source of error, owing to an increase in the total gas volume due to water vapor. At any rate, the carbon dioxide ratio was so determined and compared with that in the fermentation tube.

The gas produced in the constricted fermentation tube was first measured with Frost's gasometer and recorded in percentage of the total length of the closed arm occupied by the gas. Immediately following, the open branch was completely filled with 10% NaOH and stoppered with a rubber stopper in such a way as not to inclose any air. The gas collected in the closed arm was then rocked into contact, shaken

TABLE 2  
A COMPARISON OF CARBON DIOXIDE RATIOS OBTAINED FROM SEALED FLASKS AND FROM THE CONSTRICTED FERMENTATION TUBE WITH MARBLE SEAL

Culture	Experiment Number	Period of Incubation, Hours	Flask*		Constricted Fermentation Tube*	
			A	B	A	B
<i>B. welchii</i> 2	1	48	32.8	33.0	35	38
	2	64	29.9	29.9	30	36
	3	48	30.9	29.6	39	36
	4	48	31.5	32.7	36	34
<i>B. welchii</i> 20	5	64	26.0	27.0	34	33
	6	48	29.0	28.8	33	35
	7	48	29.5	30.6	29	10
<i>B. welchii</i> 36	8	64	16.9	18.2	19	20
	9	48	8.8	9.9	6	5
	10	48	11.0	9.0	16	10
<i>Bact. coli</i>	11	48	53.0	52.0	36	21
	12	48	49.0	51.0	39	37

\* The figures indicate percentage of carbon dioxide in the total amount of gas produced.

thoroughly, and rocked back into the closed arm; the large rubber stopper was then removed, the residual gas was measured and the difference estimated as carbon dioxide in percentage of the total gas production. The unselected results of these experiments are recorded in table 2 for 3 carefully purified and identified strains of *B. welchii* and a single strain of *Bact. coli*.

Comparing the duplicates in each experiment as summarized in the foregoing, relatively few discrepancies are seen although certain exceptions occur as in exper. 7, 10 and 11. These are inexplicable except on an assumption of possible differences in rate of growth, such as might be attributed to variations in size of inocula or possibly to unknown factors. Larger discrepancies occurred in the tubes than in the flasks.

It is curious that, with the exception of exper. 7 and 9, the carbon dioxide ratios for *B. welchii* averaged higher in the tubes than in the flasks. This difference may have been attributable to an error introduced in the water vapor as suggested, but that could scarcely be an adequate explanation of the result for *Bact. coli* where the opposite result was obtained in the two experiments tried.

Of the 3 strains of *B. welchii*, culture 36 always gave a lower carbon dioxide ratio than the other 2. It so happens that this culture is considerably more pathogenic for guinea-pigs also, although cross protection experiments performed with these strains showed that the 3 are homologous in antigenic action.

The differences in consecutive experiments merely indicate the reticence which should be exercised in attaching any absolute importance to the values recorded. They illustrate some of the possible fallacies in gas ratio records as determined either by the simple means of fermentation tubes or the more elaborate methods of gas analysis.

This conclusion is supported by inspection of table 3 which summarizes the data obtained in an experiment to determine whether among the species available for the test certain ones present especial difficulties in the interpretation of the criteria of fermentation on a medium presumably sugar-free and on the same medium plus 1% glucose. The medium used was 2% peptone meat infusion broth which had been fermented out with *B. welchii* for 6 days. The writer has engaged with Mr. S. B. Randall in a study of the comparative value of *B. welchii* and *Bact. coli* for this purpose, which indicates certain points of superiority in *B. welchii*.<sup>13</sup> In preparing this medium  $H^+$  ion concentration and titratable acidity curves were carefully followed during the fermentation and reached their maxima from 24-30 hours after inoculation. By all ordinary conceptions such a medium should be free from sugar. No further adjustment of reaction was required since after heating to kill *B. welchii* the medium had a  $P_H$  value equal to 7. The lot was divided into 2 portions, to one of which 1% glucose was added. These portions were tubed in the new constricted fermentation tubes. Sterilization performed was by the intermittent method. The mediums were inoculated from new brain cultures and incubated at 37 C. Daily readings were made for total gas production using Frost's gasometer and recorded in percentage of the total length of the closed arm occupied by the gas. No readings were made on the fifth day.



On the sixth day tests for aerobic contamination were made from the open branch by spotting on plain agar and incubating aerobically. No aerobic contamination occurred. The tests for reaction change were made from the open branch also, for in all cases the broth in the

TABLE 3  
GAS AND ACID PRODUCTION OF CERTAIN ANAEROBES IN SUGAR-FREE BROTH AND IN  
GLUCOSE BROTH

Culture Number	Culture Name	Sugar-Free Broth			Sugar-Free Broth Plus 1% Glucose		
		Total Gas*	Carbon Dioxide	Reaction Change	Total Gas	Carbon Dioxide	Reaction Change
Control	Uninoculated	Trace	..	None	Trace	..	None
1	<i>B. tetani</i>	10	10	Alkaline	12	10	Alkaline
		12			12		
		12			12		
		13			13		
2	<i>B. welchii</i>	Trace	0	None	21	18	Acid
		5			25		
		5			30		
		5			25		
		5			22		
4	<i>Vibrio septique</i>	Trace	0	None	15	20	Acid
		5			13		
		5			30		
		7			30		
		6			25		
8A	<i>B. botulinus</i>	Trace	..	Alkaline	Trace	100	Acid
		Trace			5		
		Trace			12		
		Trace			15		
10	<i>B. sporogenes</i>	Trace	..	Alkaline	3	100	Acid
		Trace			12		
		Trace			17		
		Trace			25		
		Trace			30		
50	<i>B. bifermentans</i>	Trace	..	Alkaline	Trace	61	Acid
		Trace			20		
		Trace			30		
		Trace			35		
		Trace			38		

\* The numbers indicate daily readings in percentages of the length of the closed arm occupied by the gas. The percentage of carbon dioxide and the reaction change were determined on the sixth day.

open arm had become turbid by admixture from the anaerobic portion of the tube. Since it is known that both the titratable acidity and  $H^+$  ion concentration of the open arm and of the closed arm differ, and since that of the latter was inaccessible in this experiment without disturbing the gas, in which the principal interest in this experiment lay.

only a qualitative determination of the change in reaction was made by comparison with the broth of the uninoculated control, in a porcelain test plate, using brom-thymol-blue ( $P_H$  range 6.0—7.6) as the indicator.

Only the nonputrefactive anaerobes, *B. welchii*, and *Vibrio septique*, and the faintly putrefactive *B. tetani*, produced appreciable gas in the sugar-free broth. That of *B. tetani* was the same in quantity from sugar-free and glucose broth, as might be expected on the theory that this organism ferments no sugar. This is supported by the alkaline reaction of both lots of medium. Curiously there was an appreciable quantity of carbon dioxide in both, so for *B. tetani* it is impossible that carbon dioxide may be considered a criterion of fermentation. Both *B. welchii* and *Vibrio septique* produced more gas in glucose broth than in sugar-free broth and while the former contained carbon dioxide, the latter contained none. The terminal reaction of the sugar broth was acid while that of the sugar-free broth could not be distinguished from the uninoculated control. For these organisms both carbon dioxide and acid might be considered criteria of fermentation; total gas might be so considered only with reservations as to quantity.

The putrefactive anaerobes, *B. botulism*, *B. sporogenes*, and *B. bifermentans* produced no visible gas in the sugar-free broth, and the terminal reaction was strongly alkaline, much more so than the tetanus cultures. As observed in other experiments not here reported, these organisms, which are known to attack glucose, produced abundant gas from glucose broth, but the rate of production was considerably less rapid than that of the nonputrefactive anaerobes. And here, as elsewhere in our notes, we observe that the percentage of carbon dioxide tends to be considerably higher for the putrefactive anaerobes than for more versatily fermentative nonputrefactive micro-organisms.

If we are to regard the gas produced by certain fermentative organisms in sugar-free broth as derived from proteins, we are in somewhat of a dilemma in finding that the most highly putrefactive anaerobes do not offer this difficulty in the interpretation of fermentation reactions. The only consistent phenomenon in the experiment is the reaction change which throughout permits the correlation of acidity with other evidence of fermentation.

An experiment was then planned to determine the effect of variation in the peptone buffer content of a medium. A lot of meat infusion was divided into 2 parts. To one was added 0.5% NaCl and 0.5% Parke Davis and Co. peptone; to the other was added 0.5% NaCl

and 5% Parke Davis and Co. peptone. Each was fermented out with *B. welchii* well past the turning point in the PH curve. The reactions were adusted to PH 7 and from each, eight of the new constricted fermentation tubes were filled. The remainder of each lot of medium was again divided, 1% Pfanstiehl glucose C. P. being dissolved in one, 1% Baker's lactose C. P. to the other. These were likewise tubed and all were then sterilized by the intermittent method in the Arnold sterilizer.

These mediums were then inoculated from fresh brain cultures of three carefully purified and identified strains of anaerobes representing not only the extremes in fermentative action among bacteria but also wide differences in proteolytic action. Thus *B. tetani* ferments no carbohydrates, *B. botulinus* ferments glucose but not lactose, while *B. welchii* ferments both glucose and lactose. And in the ascending order of their proteolytic action they stand *B. welchii*, *B. tetani*, *B. botulinus*. It was thought that a comparison of the action of these organisms on mediums of similar protein but of differing carbohydrate content and on mediums of similar carbohydrate but differing protein content, with respect to total gas production, carbon dioxide ratio, titer, and direction of reaction change, should give the clue to the true criterion of fermentation. The results are shown in table 4.

Each test was done in duplicate in order that one tube might serve for the determination of the carbon dioxide ratio, the other for the acidity tests. The tubes were incubated at 37 C, for 4 days during which daily observations of turbidity and total gas formation were made. Aerobic contamination tests made by subcultures on plain agar plates showed only a single contamination in one of the uninoculated controls.

The experiment was at once disappointing and instructive in failing to show, in the case of any of these organisms any notable gas production, during 4 days incubation at 37 C, in any single tube containing a sugar not definitely fermented.

*B. tetani* failed to grow in the tubes containing only 0.5% peptone and produced only traces of gas even in the 5% peptone mediums, although there was abundant evidence of growth in the turbidity of the cultures after the first day of incubation. *B. tetani* may be designated as an obligately putrefactive organism, although its putrefactive properties are feeble. *B. botulinus* which, with the exception of one tube of sugar-free medium, grew in both the 0.5% and the 5% peptone medium, likewise produced only a trace of gas in the latter in the

TABLE 4  
RELATION OF BUFFER CONTENT TO ACTION OF *B. TETANI*, *B. ROTULINUS*, AND *B. WELCHII*  
ON GLUCOSE, LACTOSE, AND SUGAR-FREE BROTH

	Peptone	Tube	Total Gas			%CO <sub>2</sub>	Titer*	Reaction Change
<b>B. tetani 1</b>								
Sugar-free.....	0.5%	a	—	—	—	—	—0.1	None
		b	—	—	—	—	—0.1	None
	5.0%	a	t	tr	tr	tr	—	—0.2
		b	t	tr	tr	tr	—	—0.1
1% glucose.....	0.5%	a	—	—	—	—	+0.1	None
		b	—	—	—	—	+0.1	None
	5.0%	a	t	tr	tr	tr	—	+0.9
		b	t	tr	tr	tr	—	+0.9
1% lactose.....	0.5%	a	—	—	—	—	0	None
		b	—	—	—	—	+0.1	None
	5.0%	a	t	tr	tr	tr	—	+0.4
		b	t	tr	tr	tr	—	+0.4
<b>B. botulinus 8A</b>								
Sugar-free.....	0.5%	a	—	t	t	t	—	—0.1
		b	—	—	—	—	—	—0.1
	5.0%	a	t	tr	tr	tr	—	+0.2
		b	t	tr	tr	tr	—	+0.3
1% glucose.....	0.5%	a	5	40	45	40	33	—
		b	5	40	15	16	—	—
	5.0%	a	tr	30	50	50	75	—
		b	—	10	10	12	—	+3.4
1% lactose.....	0.5%	a	t	t	t	t	—	+0.1
		b	t	t	t	t	—	+0.1
	5.0%	a	t	tr	tr	tr	—	+0.8
		b	t	tr	tr	tr	—	+0.9
<b>B. welchii 2</b>								
Sugar-free.....	0.5%	a	—	—	—	—	—	—0.1
		b	—	—	—	—	—	—0.1
	5.0%	a	—	—	—	—	—	+0.3
		b	—	—	—	—	—	+0.2
1% glucose.....	0.5%	a	25	20	20	20	tr	—
		b	12	15	15	15	—	+0.9
	5.0%	a	30	30	30	30	—	+4.1
		b	35	30	30	30	20	—
1% lactose.....	0.5%	a	30	25	25	25	tr	—
		b	30	25	25	25	—	+1.7
	5.0%	a	50	50	50	40	—	+3.6
		b	60	50	60	55	20	—
<b>Control uninoculated</b>								
Sugar-free.....	0.5%	a	—	—	—	—	—	—0.1
		b	—	—	—	—	—	—0.1
	5.0%	a	—	—	—	—	—	+0.2
		b	—	—	—	—	—	+0.3
1% glucose.....	0.5%	a	—	—	—	—	—	+0.1
		b	—	—	—	—	—	+0.2
	5.0%	a	—	—	—	—	—	+1.2
		b	—	—	—	t	—	Contaminated
1% lactose.....	0.5%	a	—	—	—	—	0	None
		b	—	—	—	—	+0.1	None
	5.0%	a	—	—	—	—	0.5	None
		b	—	—	—	—	0.8	None

\* Titer to brom-thymol-blue  $P_H = 7$ ; — = no growth; t = turbidity indicating growth; gas production indicated by tr (trace) or numbers which refer to percentage of closed arm occupied in the case of total gas, and to percentage of total gas in case of carbon dioxide. Titration values indicate percentage normal acid (+) or alkali (—).

sugar-free and lactose cultures. *B. welchii* failed to give any evidence of growth in sugar-free broth; it may be designated as an obligately fermentative micro-organism. The results, which would admit total gas as a criterion of fermentation, cannot be reconciled with those shown in table 3 although they appear to be remarkably consistent throughout each separate experiment.

Certain points in the observation of the fermented tubes are significant. Again we note the slower but ultimately greater gas production of the putrefactive *B. botulinus* as compared with the saccharolytic *B. welchii*, in their action on glucose, although in each set with *B. botulinus* there were discrepancies in the duplicates which cannot be accounted for. Also with both organisms the total gas production was greater with 5.0% peptone. These phenomena were almost certainly due to the buffer action of the peptone. The total gas production with *B. botulinus* was greater possibly because of the increase of buffer capacity through proteolysis.

The equipment was not sufficiently delicate to measure the carbon dioxide in those tubes showing only traces of total gas. In all tubes showing considerable total gas, the carbon dioxide ratio was, as always before, higher with the proteolytic organism in similar mediums and also distinctly higher with both the proteolytic *B. botulinus* and the saccharolytic *B. welchii* in the medium rich with peptone. While this might be thought to be due to the more vigorous growth, it is not easy to see why the ratio of carbon dioxide to other gases should be so affected. *B. welchii* produced only the barest trace of carbon dioxide in the lightly buffered medium either with glucose or lactose.

The reaction changes were determined by the test plate method already described, using culture fluid from the small branch after removing the rubber stopper. Only the titer to brom-thymol-blue was ascertained.

In sugar-free medium there was no significant change either in  $H^+$  ion concentration or in titer, as compared with the uninoculated controls, except in the case of *B. tetani* in 5% peptone medium; this was slightly alkaline, both in its reaction change and in titer. A similar change occurred with *B. tetani* in the medium plus glucose and lactose respectively. It is apparent that the introduction of the sugars brought about an increase in titratable acidity in these mediums, but the direction of reaction change due to *B. tetani* was the same in all.

We were unable, in this experiment, to note any distinct alkaline change in the sugar-free broth inoculated with *B. botulinus*, as in table 3, the lactose broth also had remained practically stationary in reaction.

Turning to those cultures in which considerable gas was formed, all had increased markedly in  $H^+$  ion concentration and in titratable acidity. The latter was especially notable in the richly buffered medium, but the former was equally distinct in the 0.5% peptone broth as would be expected. It is apparent that a high titratable acidity is correlated with a high buffer content which may be conceived to operate by permitting the more complete hydrolysis of the sugars.

This experiment alone would admit the validity as criteria of fermentation of: (1) total gas formation amounting to over 10% of the closed branch within 48 hours, (2) a distinct increase in titratable acidity, and (3) a distinct increase in  $H^+$  ion concentration. It would deny the validity of carbon dioxide as a criterion in view of its reduction to the vanishing point in a medium containing little protein which still supports active fermentation of suitable sugars by *B. welchii*. The absence of carbon dioxide from these tests in which fermentation is otherwise indicated and its presence in the tetanus cultures of table 3 in which there is no other evidence of fermentation eliminates it from further consideration in this paper.

We have remaining the criteria of total gas production, titratable acidity, and increased  $H^+$  ion concentration.

The perfect correlation of vigorous gas production and acidity changes in table 4 as contrasted with table 3 might suggest that the supposedly sugar-free medium of table 3 was actually not sugar-free, judging from the growth of *B. welchii* and *Vibrio septique* in it. But the fact that *B. tetani* produces gas in any medium in which it grows can lead to only one of two conclusions, either gas is not a reliable criterion of fermentation or the tetanus bacillus is a fermentative organism, and the latter supposition is not supportable.

Thus, while gas production is a criterion of fermentation for certain anaerobic as well as aerobic micro-organisms, there are organisms such as *B. tetani*, for which it is not a criterion, and its utility is limited to those bacteria which do not produce it in supposedly sugar-free medium.

The experiments up to this point indicated the reliability of vigorous gas production as a suitable criterion of fermentation when the control tests without sugar show only slight or no gas production. One

cannot always insist that such control tests should show evidence of growth in view of the failure of certain saccharolytic organisms to grow in the absence of fermentable carbohydrates; whether such gas as is produced should be attributed to proteolysis alone depends on the organism. *B. tetani* appears to produce gas by proteolysis; most other proteolytic organisms do not. The anaerobes which give the most difficulty in the interpretation of fermentation in tests in which gas is the criterion are to be found among the saccharolytic groups which are relatively less active protein splitters. The inevitable conclusion is that gas formation in the "sugar-free" control in such cases is due to sugar which was not fermented out of the medium. This is likely to be the case especially when *Bact. coli* or *Bact. saccharolyte* are used for this purpose. It is less likely to be true when *B. welchii* is used.<sup>13</sup> Meat infusion peptone broth fermented out with *B. welchii* gives little difficulty in utilizing vigorous gas production as a criterion of fermentation for even with 5% peptone abundant gas production does not occur except in the presence of fermentable sugars.

The conclusions as to the acceptability of gas as a criterion of fermentation relate purely to peptone medium prepared as described. The possibility that proteolytic organisms may produce gas from native protein, such as casein, serum or egg albumin in the absence of fermentable sugars remains for more detailed study. The point is important because most of the present accepted records<sup>24</sup> of anaerobic fermentation reactions are based on the work of Henry,<sup>18</sup> who used egg albumin as an indicator for acid production in a casein digest medium. As for the casein in milk, our results in table 1 show a small amount of gas production by certain putrefactive anaerobes, but this may have been derived from the fermentation of the small amount of monosaccharide which Smith<sup>25</sup> and Jones<sup>26</sup> have shown to exist in milk, or by the indirect hydrolysis of the lactose. We have no data on the possibility of gas production from sugar-free casein, but it is well known that blood serum contains sugar.<sup>27</sup> Hammersten (p. 601) also refers to the presence of a fermentable carbohydrate in egg albumin and points out that "the protein substances of the white of egg behave like glycoproteins, as they all yield glucosamine." The addition of these substances

<sup>24</sup> Reports of the Committee upon Anaerobic Bacteria and Infections, Medical Research Committee—Special Report Series, No. 12, 1917, and No. 39, 1919; also footnote 7.

<sup>25</sup> Jour. Boston Soc. Med. Sc., 1897, 2, p. 236.

<sup>26</sup> Jour. Infect. Dis., 1914, 15, p. 357.

<sup>27</sup> Hammersten: Textbook of Physiological Chemistry, 1912, p. 256.

to sugar-free mediums for the purpose of enrichment or as indicators may confuse the readings in the controls through the addition of fermentable sugars.

There is a possibility that this is true also of agar, which, consisting mainly of d-galactose, a carbohydrate belonging to the group of pentosans, is subject to hydrolysis by autoclaving.<sup>28</sup> Fellers<sup>29</sup> quoted Tollens and Bourgeois "Hydrates de carbone" as saying "lactose and mixtures of glucoses have been crystallized from agar treated with dilute acids," although Fellers was unable to repeat this and failed to demonstrate gas formation by *Bact. coli* and yeasts in fermentation tubes.

That serum, egg albumin and agar may be used in aerobic fermentation tests without causing discrepancies means nothing in the study of anaerobic micro-organisms whose metabolism is so much more vigorous.

#### ACID AS A CRITERION OF ANAEROBIC FERMENTATION

All gas-forming aerobes produce also some acid and many fermentative aerobes produce acid without gas; acid is even more widely used than gas as a single criterion of aerobic fermentation. For many years it was customary to utilize merely the qualitative fact of acid production; then it became the fashion to measure the titratable acidity, and now the  $H^+$  ion concentration, usually expressed in terms of  $P_H$  value, is most popular as a criterion of acidity. Each of these steps has marked a development in our conception of the fundamental chemistry of the fermentative reaction.

#### TITRATABLE ACIDITY

The invalidity of titratable acidity as a criterion of direct lactose fermentation in milk by anaerobes has been pointed out in connection with table 1 which shows that the nonfermenting *B. botulinus*, *B. sporogenes*, and *B. bifermentans* produced titration values similar to that of *B. welchii* which ferments lactose, whereas *Vibrio septique* and *B. chauveauii* which also ferment lactose, judging by their reaction changes, produced lower titration values. Unquestionably the high titration of milk cultures of the putrefactive anaerobes are dependent in part on the increased buffer capacity of the hydrolyzed casein, although the indirect fermentation of the lactose may also be concerned.

<sup>28</sup> Noyes: Science, 1916, 44, p. 797.

<sup>29</sup> Soil Science, 1916, 2, p. 255; Jour. Ind. and Eng. Chem., 1916, 8, p. 1128.



Owing to certain technical difficulties that we hope to overcome, we have not yet been able to find out whether sugar-free casein shows a similar increase in buffer capacity on proteolysis by these organisms. Attention is directed to the influence of coagulable protein in the titration curve of *B. welchii*.<sup>13</sup>

Peptone, even in 5% concentration, introduces no difficulty of interpretation for the organisms tested, as may be seen in table 4. In fact, there was a decrease in titratable acidity with *B. tetani* in all the highly buffered mediums, and while there was no notable decrease in titer with *B. botulinus* in lactose peptone broth, there was also no evidence of an indirect attack on lactose, such as may be observed in milk. On the contrary, there is in the data displayed in table 4, a perfect correlation of abundant gas formation, high titration, and increased  $H^+$  ion concentration in the action of *B. botulinus* on glucose, and of *B. welchii* on both glucose and lactose.

#### HYDROGEN-ION CONCENTRATION

All in all, reaction change in the direction of increased  $H^+$  ion concentration seems to be the most constant criterion of fermentation. It has not seemed necessary to the writer in the present investigation to determine the actual  $P_H$  values, though none may dispute a degree of satisfaction that attends the contemplation of a numerical record. Yet a serious question may be raised as to the absolute value of such records before we have learned to use qualitative methods with assurance. It is more important for present purposes to record and interpret the direction of change in  $H^+$  ion concentration than it is to record those quasi-absolute values represented in titration and  $P_H$  determinations in "standard" mediums incubated for a "standard" time at a "standard" temperature without any clear recognition of whether the sugar present is an excess or less than an excess, or what the relation of sugar to protein may be, or what the influence of time.

In addition to its uniformity as a criterion of fermentation, an increase in  $H^+$  ion concentration has the added advantage over gas as a criterion, that, if brought about by the presence of a relatively small amount, i. e., less than an excess, of an unsuspected fermentable carbohydrate, the exhaustion of the sugar will effect a reversion, whereas gas is more persistent. There is therefore less tendency to confusion in the controls, and full reliance may be placed in a negative result showing alkalinity even with slight gas production.

Among recent students of anaerobes both qualitative and quantitative tests for acidity have been employed. Distaso and Martinez <sup>30</sup> in 1913 used a deep semisolid agar medium with litmus as the indicator. This dye is decolorized in the depths by the growth of anaerobes, but shows the direction of the reaction change at the surface in contact with air. Meyer <sup>14</sup> Simonds <sup>5</sup> and Esty <sup>5</sup> have left records in terms of titratable acidity using phenolphthalein as the indicator. But the most recent trend appears to be toward the use of purely qualitative tests as in the work of Robertson, <sup>6, 15</sup> Henry <sup>18</sup> and Adamson. <sup>31</sup> Henry, <sup>18</sup> whose insight into the problem has already been mentioned, pointed out that for some organisms, peptone solution lacks sufficient nutriment, and he employed a neutral pancreatic digest of casein with egg albumin as an indicator, whose coagulation shows the production of acid. In doubtful cases litmus might be added; its inclusion previous to incubation was found to be valueless because of its decolorization under the paraffin used as a seal.

At one time during the course of the present investigation and before the point of view expressed in the foregoing paragraphs was reached, the writer hoped to find an indicator that might be added to the mediums in order to enable the investigator to follow the  $H^+$  ion changes in the closed branch of the tube without disturbing the culture. Such an indicator should have the following characteristics:

1. It should show distinct color changes throughout a relatively wide range of  $P_H$  values on both the acid and the alkaline sides of the neutral point, i. e.,  $P_H=7.0$ .

2. It should be neither decolorized nor destroyed by sterilization under anaerobic conditions.

3. It should be noninhibitive in the concentration required as an efficient indicator.

4. It should not be decolorized or destroyed by the growth of anaerobic organisms.

These requirements have not been found combined in a single indicator or combination of dyes. Table 5 presents the findings on a few whose known  $H^+$  ion concentration ranges suggested their possible usefulness. A number of dyes, whose range lies wholly on one side of the neutral point, were tested with the possibility in view that they

<sup>30</sup> Compt. rend. Soc. de biol., 1913, 75, p. 201.

<sup>31</sup> Jour. Path. & Bacteriol., 1919, 22, p. 345.

might be combined with another having a complementary range on the other side of the neutral point. The ranges were determined in buffer solutions of known  $P_H$  values.

The azolitmin was an old sample of Merck's. The phenol red, brom-thymol-blue, cresol red, and brom-cresol purple, were made by Hynson, Westcott and Company. The source of the China blue used separately is unknown. The China blue-phenol red mixture was made up according to Morishima's formula.<sup>32</sup> The China-blue rosolic-red

TABLE 5

A STUDY OF CERTAIN DYES WITH REFERENCE TO THEIR POSSIBLE UTILITY AS DIRECT INDICATORS OF  $H^+$  ION CHANGES IN ANAEROBIC CULTURES

Indicator	$P_H$ Range	Concentration	B. welchii 2 in Glucose Broth			B. tetani 1 in Sugar-free Broth		
			Decolorization by Heat	Growth	Decolorization by Growth	Decolorization by Heat	Growth	Decolorization by Growth
Azolitmin.....	6.8-7.8	1:2,000	+	+	+	+	+	+
Phenol red.....	6.7-8.4	1:100,000	—	+	Permanent	—	+	Partial
China blue.....	5.0-7.0	1:20,000	—	+	Permanent	—	—	—
Phenol red, china blue	5.0-8.4	P.R. 1:100,000 C.B. 1:10,000	—	+	Permanent	—	—	—
China blue, rosolic acid	Not determined	C.B. 1:40,000 R.A. 1:20,000	—	+	Permanent	—	—	—
Brom-thymol-blue....	6.0-7.6	1:25,000	+	+	Slow but permanent	+	—	—
Cresol red.....	7.2-8.8	1:50,000	+	+	Negative, 48 hours	+	+	Negative, 48 hours
Hematin.....	6.0-8.2	1:20,000	—	—	—	—	—	—
Acid fuchsin.....	6.4-8.2	1:20,000	—	+	Slow but permanent	—	+	Slow but permanent
Brom-cresol purple....	5.2-6.8	1:50,000	—	+	Permanent	—	—	—
Neutral red.....	7.6-8.6	1:10,000	—	+	Permanent	—	—	—

\* Temporary: Color regained aerobically on cooling, not anaerobically except on exposure to air

† Partial: Color regained aerobically and anaerobically on cooling.

mixture was kindly supplied by Dr. Bronfenbrenner<sup>33</sup> and used according to the directions on the bottle. The hematein and neutral red were old samples of Grubler's. The acid fuchsin was Harmer's and was used in a concentration equivalent to that in Andrade's indicator.

The study of these dyes was begun by determining the limit of usefulness in graded dilutions of N/20 lactic acid and N/20 ammonia.

<sup>32</sup> Jour. Infect. Dis., 1920, 26, p. 43.

<sup>33</sup> Jour. Med. Research, 1918, 34, p. 25.

which were selected because they are the chief agents that affect the reaction in bacterial cultures. After determining the limit of useful dilution of dye, the useful range of each was determined in a graded series of standard buffer solution. The first tests were made in constricted tubes with marble seals with glucose broth ( $P_H=7.4$ ) containing these dyes in the indicated concentrations.

The tubes were examined for decolorization by heat after each intermittent sterilization. In such tests the constricted tube provides both aerobic and anaerobic conditions so that the return of color that occurs with some dyes when their leuco-bases are exposed to the air may be readily observed above the seal and contrasted with the failure of recolorization below the seal.

Only axolitmin failed to meet the requirement of not decolorizing during sterilization for while brom-thymol-blue and cresol-red were partially decolorized by heating, they completely regained their color both aerobically and anaerobically on cooling.

*B. welchii* was selected for its active fermentative properties as the test organism in glucose broth. Of the dyes tested in glucose broth, only hematin proved inhibitive. In all the rest growth was shown by turbidity and abundant gas formation at 37 C, in less than 24 hours.

Cresol red was the only dye not decolorized by growth of *B. welchii* in glucose broth; it is of no value as an indicator of acid, however, on account of its range which lies on the alkaline side, but it might prove to be a satisfactory component of a combination with some other dye.

Both brom-thymol-blue and acid fuchsin have a correct range, but they were destroyed by the growth of the organism. Neither exposure to air nor the addition of either acids or bases caused either of them to regain their indicator properties after 48 hours' incubation. These two dyes were less rapidly destroyed however than phenol red, China blue, China blue-rosolic acid, phenol red China blue, brom-cresol-purple and neutral red, all of which were completely and permanently decolorized at 24 hours.

Azolitmin alone of all the dyes sensitive to both acids and alkalines at the neutral point was not destroyed by the growth of *B. welchii* in glucose broth, although it is of no value for the direct determination of  $H^+$  ion concentration owing to its decolorization under anaerobic conditions both by heat and by growth. Such decolorized azolitmin retains its indicator properties when exposed to air, however. Moreover,

the medium above the seal was not decolorized and served in situ as a valuable qualitative indicator of increased  $H^+$  ion concentration.

The question was raised as to whether decolorization of these dyes might not in itself be regarded as a criterion of fermentation. This point was put to test using *B. tetani*, a nonfermentative organism, in sugar-free meat infusion broth fermented out with *B. welchii*.<sup>33</sup> Although performed at a later time, the results of this experiment also are presented in table 5. So far as they go, they show that decolorization is not correlated with fermentation.

It was impossible to secure satisfactory growth of *B. tetani* in sugar-free broth with brom-thymol-blue, brom-cresol-purple or neutral red, although the tubes were reinoculated after 48 hours' incubation and reincubated. Otherwise the results were comparable with those with *B. welchii* in glucose broth.

Azolitmin behaved exactly as in glucose broth with *B. welchii*; it was decolorized below the marble seal and not above. When tested after 48 hours' incubation the decolorized dye regained its color (blue) on exposure to air in a test plate and was still sensitive to the addition of lactic acid.

Acid fuchsin at 48 hours was completely and permanently decolorized; it failed to react to the addition of lactic acid.

Phenol red at 48 hours was yellow in the culture tube, although a test of the solution with brom-thymol-blue showed distinct alkalinity. The phenol red was only partially decolorized, however, as it still reacted to strong lactic acid.

Cresol red appeared not to be destroyed in 48 hours, but its indicator properties in the tube were not altogether satisfactory.

It is curious that while the direct and progressive determination of  $H^+$  ion concentration changes wrought by certain facultatively anaerobic bacteria may be made as noted by Bronfenbrenner,<sup>33</sup> Morishima,<sup>32</sup> Conn and Hucker<sup>34</sup> and Medalia,<sup>35</sup> it seems impossible to utilize these dyes similarly in the study of obligate anaerobes such as *B. welchii* and *B. tetani*. The outcome of our admittedly fragmentary search for an indicator for the direct determination of  $H^+$  ion concentration has thus been disappointing. Only a qualitative determination of the direction of  $H^+$  ion concentration change may be made using the dye in the medium, and for this litmus has proved so far the best indicator.

<sup>34</sup> Jour. Bacteriol., 1920, 5, p. 433.

<sup>35</sup> Ibid., 1920, 5, p. 441.

Litmus must be used in such a way as to expose it both to the action of the air to avoid decolorization and to the influence of the acid formed by the anaerobes. This may be done in deep agar according to the method of Distaso and Martinez<sup>30</sup> or in the constricted tube with marble seal. The writer has used both methods successfully in qualitative tests but prefers the latter in spite of its demand for a rather larger quantity of medium.

For quantitative tests it is still necessary to remove the culture fluid from the container. Our present methods leave much to be desired in this direction; the elaborate devices invented with this end in view by Wolf and Harris<sup>23</sup> have already been mentioned. For purposes of following simply the titratable acidity and  $H^+$  ion curves the method used by Randall and Hall<sup>13</sup> is quite satisfactory.

#### THE PRESENT STATUS OF THE PROBLEMS OF ANAEROBIC FERMENTATION TESTS

The experiments here outlined suggest the following observations:

The vigorous production of gas by anaerobes is generally to be regarded as an indication of fermentation. However, two sets of facts may confuse the utilization of gas as a criterion of fermentation in special cases; first, the production of gas from simple sugars present in the medium and not removed by the method of sugar removal generally employed, i. e., by fermentation with *Bact. coli*, and second, the production of small quantities of gas from proteins by certain organisms, such as *B. tetani*, not known to have any action on sugars. The first of these difficulties may be overcome through the use of *B. welchii* instead of *Bact. coli* in the preparation of sugar-free mediums.

The production of carbon dioxide by *B. tetani* in both sugar and sugar-free mediums also makes the utilization of carbon dioxide as a criterion of fermentation untenable.

The utility of titratable acidity as a criterion of fermentation is limited to mediums not containing coagulable proteins, the hydrolysis of which by proteolytic organisms greatly increases the buffer capacity of the titrated samples.

An increase in  $H^+$  ion concentration is regarded as the best evidence of fermentation. It has the advantage over gas of nonpersistence when the protein phase of metabolism becomes dominant through exhaustion of small amounts of sugars not supposed to be included in a given test.

No means of direct quantitative determination of  $H^+$  ion concentration have been found. Certain dyes that have been so utilized in the study of aerobes are not only decolorized but destroyed by certain, possibly all, anaerobes. Litmus alone was found useful for qualitative tests. Its utilization in the constricted tube (with or without the gas collection tube) is suggested. Emphasis is laid on the comparison of all fermentation tests with uninoculated mediums and with inoculated sugar-free mediums.

## THE USE OF *B. WELCHII* IN THE PREPARATION OF SUGAR-FREE CULTURE MEDIUM

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The vigor of fermentation of *B. welchii* suggested that it might prove superior to the more commonly used aerobic organisms in the elimination of muscle sugar from mediums intended for the study of the fermentation reactions of bacteria.

That muscle sugar may confuse the interpretation of fermentation reactions has been recognized for some time. Smith,<sup>1</sup> in 1895, mentioned that the muscle sugar content might vary from a trace to 0.3% and concluded, in accord with physiologists, that it is mainly glucose since it is attacked by bacteria that do not ferment lactose or saccharose. Smith<sup>2</sup> emphasized the necessity of eliminating muscle sugar from meat infusion used in the preparation of differential carbohydrate medium, so that now it is a common laboratory procedure to use a so-called "sugar-free" medium as the basis for fermentation tests with known carbohydrates. But the details of preparation vary in different laboratories.

In some laboratories the fermenting culture is mixed with the ground meat when it is put to soak, and incubated at 37 C. overnight. In this method there is, of course, always a mixed bacterial flora. The mass is boiled to coagulate the albumins and kill the bacteria. Straining removes the former and filtration through paper most of the latter, but the product is nearly always somewhat turbid, and if clarity is desired, an absorbent, such as talc or diatomaceous earth, must be added previous to filtration. If the fermentation has proceeded too far, there is likely to be great difficulty in filtration, owing to the colloidal nature of the liquid. In such cases, acidification and reheating provide effective means for securing a filtrate, which, however, may still have to be clarified by the aid of an adsorbent as already mentioned.

In other laboratories it is customary to secure the infusion in the usual manner of cold soaking, boiling, and filtration, after which the

Received for publication May 29, 1921.

<sup>1</sup> Centralbl. f. Bakteriol., 1895, 18, p. 1.

<sup>2</sup> Wilder Quarter Century Book, 1893, p. 187; Centralbl. f. Bakteriol., 1890, 7, p. 502; Jour. Exper. Med., 1897, 2, p. 543.



muscle juice is sterilized and then fermented out with a pure culture. The objection to this practice is that the medium at this stage is deficient in nutrients so that growth of the fermenting organism is never vigorous. A better plan is to ferment out the sugar after the peptone has been added, and this procedure has the added advantage of removing traces of sugar, which may be present in the peptone also.<sup>3</sup> This has been our own procedure in the experiments shortly to be described, except in two instances in which we used sterilized meat mashes to which peptone had been added, but from which the meat had not been strained.

Most bacteriologists, following the suggestion of Smith, have used *Bact. coli* as the fermenting culture. *Bact. coli* communior ferments a greater variety of carbohydrates than most other aerobic organisms. The strain used in our work was derived from human feces and fermented with acid and gas formation, glucose, lactose, saccharose and glycerol but not inulin or salicin. It has no liquefactive action on gelatin and gives a positive methyl red test and a negative Voges-Proskauer test.

If we assume that all of the muscle sugar consists of hexoses, versatility in the fermentation reactions is not a prerequisite in the culture selected.

Thus, Dr. K. F. Meyer of the Hooper Foundation for Medical Research, San Francisco, has used a culture designated *Bact. saccharolyte* for several years in the preparation of "sugar-free" medium. The only history of this culture that is available indicates that it came from Dr. Ernst of Harvard Medical School in 1911 and was studied by Dr. Rivas at the University of Pennsylvania. It is a coli-form, gram-negative, nonsporulating rod which ferments with acid and gas production, glucose, saccharose, and salicin and gives a positive methyl red test. It does not ferment lactose, inulin or glycerol. It does not liquefy gelatin and gives a negative Voges-Proskauer test. It should be regarded as most closely related to the paratyphoid-enteritidis group of organisms, from which it differs in the fermentation of saccharose.

#### EXPERIMENTAL WORK

The experiments described in this paper deal with a comparison of *B. welchii* with *Bact. coli* and *Bact. saccharolyte*. The strain used was our *B. welchii*, No. 2, which was received from the American Museum of Natural History, May 2, 1916, under their No. B 521.

<sup>3</sup> Colway: Science, 1915, 41, p. 662.

Dr. J. P. Simonds had recovered this culture about 1912 from stools as No. 16 in his series. When received by us it was free from aerobic contamination and gave a characteristic stormy fermentation of milk, but the presence of motile rods and the production of a black deposit in brain medium indicated contamination with a putrefactive anaerobe. This organism was isolated and identified as *B. sporogenes*. It bears No. 72 in our series. The Welch bacillus was also isolated free from contamination by four times repeated streaking and picking of well isolated colonies from the surface of blood-agar slants and twice repeated picking of isolated colonies from the depths of deep meat-infusion agar.<sup>4</sup> The culture forms central spores (sparingly) that do not swell the rod, the organisms are nonmotile, coagulated albumin is not liquefied, brain is not digested and only blackened in the presence of added iron ions, gelatin is liquefied and milk is fermented with vigorous production of gas and slight acid.

In studying the preparation of "sugar-free" broth, we have considered the possible applicability of several criteria of the presence of sugar, among which may be mentioned chemical tests for glucose, acid production as indicated by changes in titer and in  $P_H$ , and in one experiment, the formation of gas by refermentation.

Tests for carbohydrates based on the principle of reduction in an alkaline solution were tried. Fehling's test and the more recently devised Folin-McEllroy reagent<sup>5</sup> were used. It was found from the first, however, that culture broth contains some ingredient or ingredients which interfere with copper reduction with either of the above reagents even in the presence of as high as 1% glucose.

On the supposition that the protein of the medium had much to do with this interference, we undertook to eliminate this by various methods. Picric acid<sup>6</sup> was quite successful as a precipitant, but the resultant deep yellow color of the filtrate would not permit its use where observation of the color changes is essential.

Precipitation of the protein by the mercury reagent of Benedict and Ostenberg<sup>7</sup> was tried next. This reagent, when applied to urine, has the advantage not only of precipitating nitrogenous compounds as completely as possible, but also other possible interfering substances such as polyphenols, and glycuronic acid. The wide range of activity of this reagent in urine suggested its efficacy when applied to culture

<sup>4</sup> Hall: Jour. Infect. Dis., 1920, 27, p. 576.

<sup>5</sup> Jour. Biol. Chem., 1918, 33, p. 513.

<sup>6</sup> Lewis and Benedict: Jour. Biol. Chem., 1915, 20, p. 61.

<sup>7</sup> Ibid., 1918, 34, p. 195.

broth. A heavy precipitate and clear filtrate were obtained from broth treated with this reagent. Subsequent tests of this filtrate with the copper reagents failed to give positive results with either "normal" or "sugar-free" broth. After the addition of a minimum of 0.1% glucose to either filtrate, however, positive results were obtained. The conclusion was that the sugar content of ordinary broth was below the range of sensitivity of either of the reagents used and that, therefore, these qualitative sugar tests were not applicable to our purpose.

We have also made some preliminary tests of the method of blood sugar analysis outlined by Folin and Wu<sup>8</sup> in reference to the sugar content of broth, but further work will be necessary for the proper interpretation of the results.

Of the various criteria that may be used to guide the preparation of sugar-free broth the changes in reaction, measured either in terms of  $P_H$  or by titration, have seemed most reliable. The peculiar advantages of each appear in the charts. Our interpretation of changes in reaction toward acidity as indicative of fermentation, i. e., sugar splitting, and of changes in reaction toward alkalinity as indicative of proteolysis, is based on Kendall's conception<sup>9</sup> that "fermentation takes precedence over putrefaction." It is our belief that the peak of a curve plotted on a basis of acidity during the growth period indicates the "sugar-free" point, and when this point is reached the incubation should be stopped, for the culture has then reached the termination of its carbohydrate metabolism and the initiation of active proteolysis.

Attention is directed to the results of cross fermentation tests using both gas and reaction changes as criteria of the incompleteness of sugar removal and to the filtrability tests of the products of fermentation by the three organisms studied, all of which point to the advantage of using *B. welchii* in the preparation of sugar-free medium.

The mediums used throughout, except in exper. 7-10, consisted of beef infusion broth prepared by soaking 5 pounds of lean ground beef in 7½ liters of distilled water overnight followed by 10 minutes' boiling. The coagulated albumin was then removed by straining under pressure and paper filtration of the liquid. To the latter were then added 2% peptone (Parke, Davis and Co.) and 0.5% NaCl. This medium was adjusted to various initial reactions as indicated in the several experiments.

<sup>8</sup> *Ibid.*, 1919, 38, p. 81.

<sup>9</sup> *Jour. Med. Research*, 1911, 25, p. 117.

The mediums were put up in lots of 400 c c in 500 c c Pyrex Erlenmeyer flasks, sterilized in the autoclave, inoculated while warm and incubated at 37 C. No special means of anaerobiosis is required under these conditions to insure abundant growth of *B. welchii*; vigorous gas production and turbidity within a few hours gave evidence of active proliferation. Aerobic subcultures on agar slants were made at different times during the incubation period for the purpose of testing the purity of the cultures in the various flasks, and in the case of *B. welchii* for the purpose of detecting possible aerobic contaminants.

The determinations of the reaction at various stages of each experiment were made as follows:

The "titratable" or "total" acidity was obtained by titrating 5 c c of the medium or culture diluted with 45 c c of distilled water, to neutrality with 0.05 Normal NaOH or HCl, using phenolphthalein as indicator. The distilled water was heated to boiling prior to use to expel any carbon dioxide present.

Determinations of the  $H^+$  ion concentration or "true" acidity were made by the colorimetric method of Clark and Lubs<sup>10</sup> and recorded in terms of  $P_H$ .

Exper. 1 (chart 1) was undertaken to secure frequent observations during the early growth cycles of *Bact. coli* and *B. welchii*. Readings were continued until no further variations appeared, i. e., for a total period of 8 days. *B. welchii* mounted to a slightly higher acid titer than *Bact. coli* and beyond the maxima, which were reached simultaneously in 8-24 hours; the titer of *Bact. coli* fell much more rapidly and reached a lower level than that of *B. welchii*. In neither case were there significant changes in titer after the 6th day and neither culture reached the neutral point of phenolphthalein.

The curves plotted on a basis of  $H^+$  concentration parallel each other more closely than do the curves plotted from the titration values. The curve for both *B. welchii* and *Bact. coli* maintain a definite flat course at  $P_H$  6.5 during 62 hours following the attainment of the maximum. It is notable that during this period, striking changes in titer were occurring, especially with *Bact. coli*. In this, as in subsequent experiments  $P_H$  6.0 seems to represent the maximum  $H^+$  ion concentration value attainable by any of the organisms studied, although in exper 7 the  $H^+$  ion concentration was somewhat higher to start with. The possible fallacy in reading colorimetric determinations at the limit of indicator range was thoroughly appreciated and avoided throughout this work.

Exper. 2 (chart 2) duplicated exper. 1 in method with a different lot of medium. The limit of acidity as measured by both titration and  $P_H$  values was practically the same as in exper. 1, but for some reason neither value fell to so low a point as in the first instance. Peculiarly, the final titer for *Bact. coli* in this experiment was the same as that for *B. welchii* in the first, but the final titer for *B. welchii* was much higher than that for *Bact. coli*.

<sup>10</sup> Jour. Bacteriol., 1917, 2, pp. 1, 109, 191.

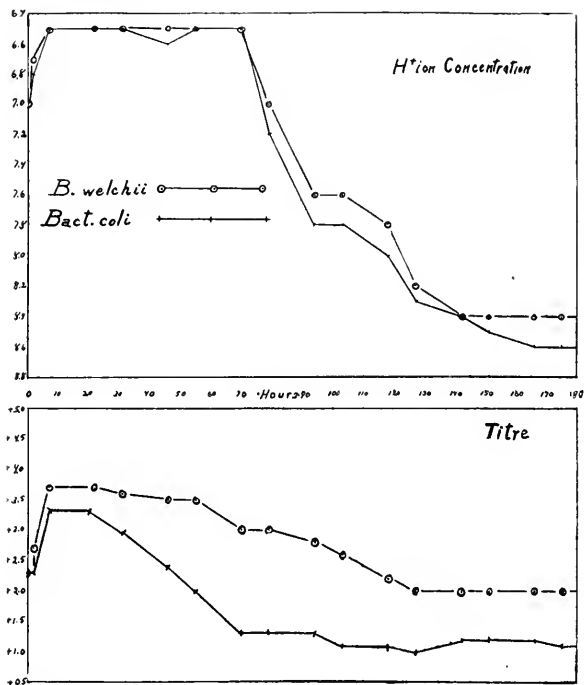


Chart 1.—Acid production of *Bact. coli* and *B. welchii* in peptone broth neutralized with NaOH, meat infusion No. 1.

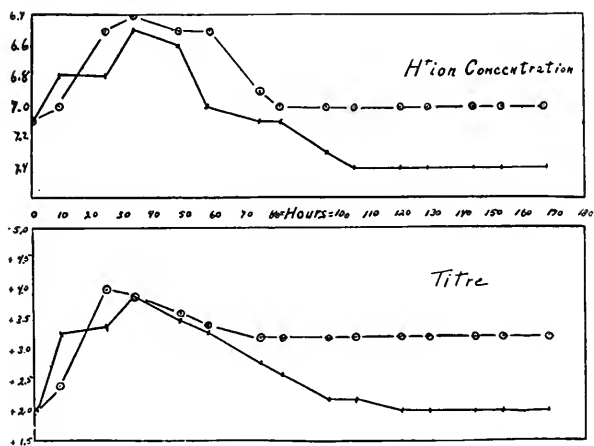


Chart 2.—Acid production of *Bact. coli* and *B. welchii* in peptone broth neutralized with NaOH, meat infusion No. 2.

The final  $P_H$  values for both organisms were also less (more acid) than in the first experiment. These differences were probably due to differences in buffer content of the two mediums.

Expt. 3 (chart 3) was carried out with broth adjusted with 0.5% magnesium carbonate, of which the excess was removed after boiling. The essen-

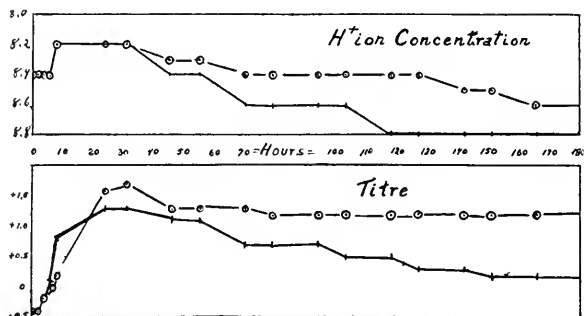


Chart 3.—Acid production of *Bact. coli* and *B. welchii* in peptone broth alkalized with magnesium carbonate; meat infusion No. 1.

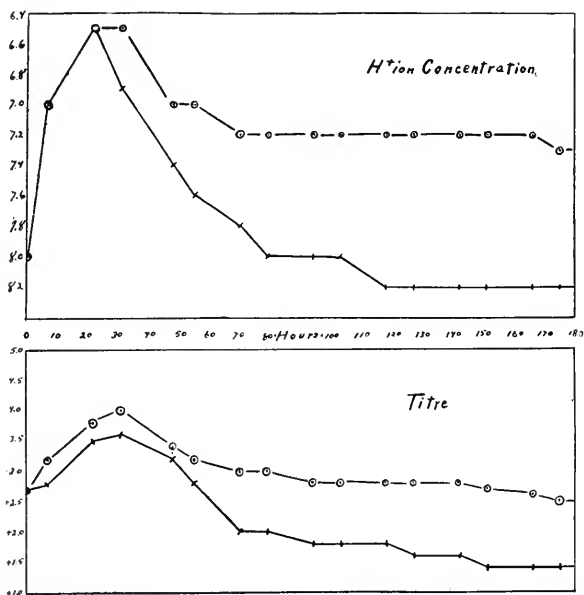


Chart 4.—Acid production of *Bact. coli* and *B. welchii* in peptone broth alkalized with NaOH; meat infusion No. 1.

tial features of the titer curves in chart 3 are similar to those of charts 1 and 2, although the peak values for both organisms are less. Again the titer for *Bact. coli* fell through a longer period and to a lower point than that of *B. welchii*. The damping effect of soluble magnesium salts is most clearly

shown in the  $P_H$  curves; those of both organisms are identical up to the 36th hour, when the acidity of both begins to fall, that of *Bact. coli* more rapidly.

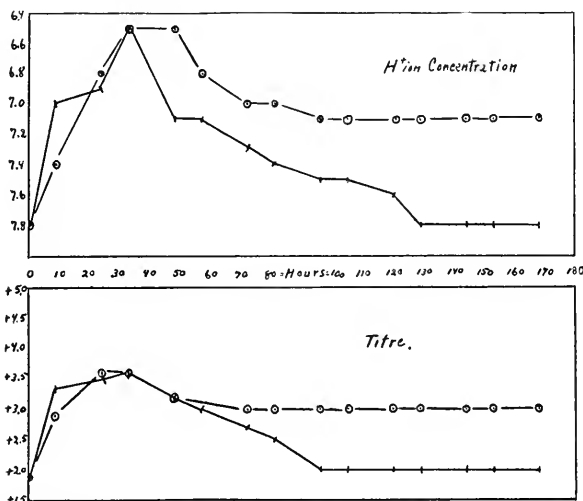


Chart 5.—Acid production of *Bact. coli* and *B. welchii* in peptone broth alkalized with NaOH; meat infusion No. 2.

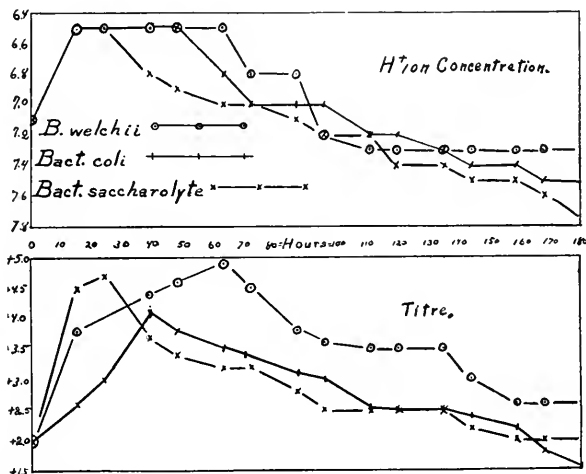


Chart 6.—Acid production of *Bact. coli*, *Bact. saccharolyte* and *B. welchii* in peptone broth alkalized with NaOH; meat infusion No. 2.

Exper. 4 (chart 4) was similar to exper 1 except that the medium, which was of the same lot as in exper. 1, was adjusted to an initial  $P_H$  value of 8.0 by means of normal NaOH without making use of magnesium carbonate

as in exper. 3. In spite of its distinct alkalinity as measured by the  $P_H$  value, this medium still titrated +2.7 in acidity to phenolphthalein. Inspection of the data shows, as outstanding features, that (1) the titrable acidity reached a slightly higher point in the case of *B. welchii*, and (2) the final  $P_H$  value and titer of *B. welchii* are not as low as are the corresponding values for *Bact. coli*.

Exper. 5 was conducted with some of the same lot of medium as exper. 2 and duplicates exper. 4 in the adjustment of initial reaction to a low  $P_H$  value with normal NaOH. Comparing charts 4 and 5, one notes the same relative

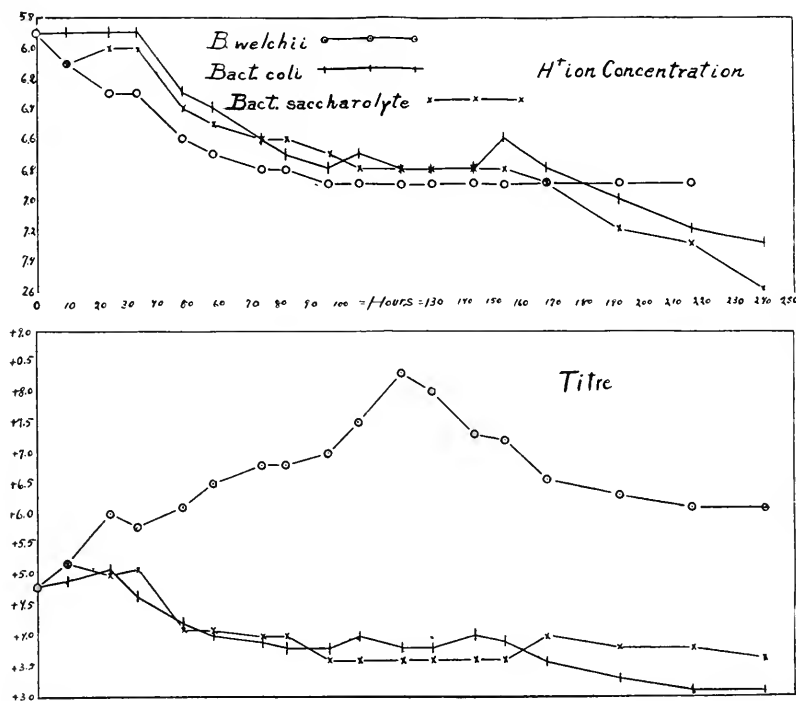


Chart 7.—Acid production of *Bact. coli*, *Bact. saccharolyte* and *B. welchii* in meat peptone mash.

differences as observed in the comparison of charts 1 and 2. Here again a possible explanation lies in the assumption of differences in buffer content of the two lots of medium.

Exper. 6 (chart 6) included *Bact. saccharolyte*. Judging from the titer curve, it reaches its maximum more quickly than either *Bact. coli* or *B. welchii*. The measured points extend higher than those of *Bact. coli* but not as high as those of *B. welchii*; the fall parallels that of *Bact. coli*; the fall of the Welch curve is slower and does not extend so low. A study of the H<sup>+</sup> ion concentration curve gives no additional data.

Exper. 7 (chart 7) was a study of the 3 cultures in a meat peptone mash. This medium was made by soaking the usual amount of ground lean meat, 5 pounds, in 7½ liters of distilled water, over night at room temperature, then



adding 2% peptone, 0.5% NaCl and sterilizing the mash in flasks in the autoclave without straining or filtering out the coagulated albumin. No adjustment of reaction was attempted; the initial titer to phenolphthalein was therefore high, +4.8, and the  $P_{H}$  value was 5.9. Owing to the removal of relatively large samples (75 cc) to provide material for the acidity tests, filtration and cross refermentation tests which were carried out in this experiment, 2 flasks of 1 liter capacity well filled for each of the aerobes and 3 for *B. welchii* were provided in order that a change might be made from flasks in which the content of medium was becoming unduly low. No irregularities in the data secured seem to be attributable to this procedure.

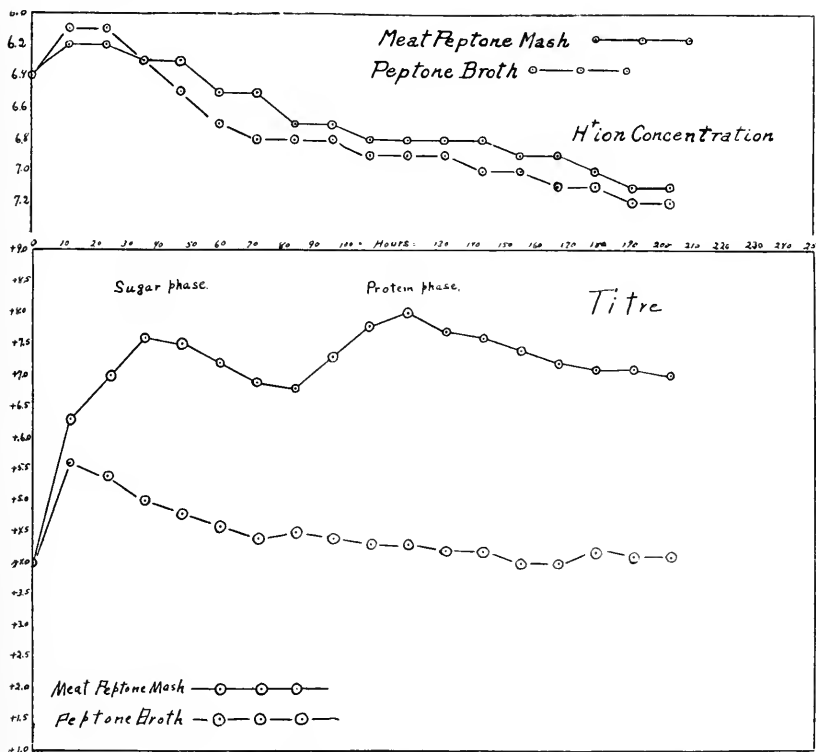


Chart 8.—Acid production of *B. welchii* in meat peptone mash and peptone broth.

The results differ from those in experiments in which the coagulated albumin had been removed in failing to show with any of the 3 organisms studied any initial increase in  $H^+$  ion concentration. This might be regarded as due to a lack of sugar in this particular lot of medium, and it is unfortunate that this experiment was not controlled with one using some of the same lot from which the coagulated albumin had been removed. We were inclined to believe the high buffer content of the medium served to mask the production of acid, or that perhaps the continuous fall in the  $H^+$  ion concentration represented

a predominance of the protein metabolism over a possible sugar metabolism due to the relative preponderance of protein in this medium. It was noted that after *Bact. coli* and *Bact. saccharolyte* had apparently reached a level between  $P_H$  6.6 and 6.8 at the 80th hour of incubation these 2 cultures began a new depression of the  $H^+$  ion concentration at the 170th hour. This did not occur with *B. welchii*. The titration curves are most striking in this experiment. After a brief initial rise in titratable acidity, the curves of all 3 organisms fell as usual. But that for *B. welchii* shortly began to ascend to a peak which it reached after the first 120 hours of incubation, when it fell distinctly.

We thought at first that the increase in titratable acidity might be attributed to an actual production of acids which failed to increase the  $H^+$  ion concentration due to their immediate neutralization by the high protein buffer content of the medium. This experiment presented the apparent enigma of a culture increasing its titratable acidity while actually becoming more alkaline as measured in terms of  $P_H$ . But the paradox disappeared when it was remembered that phenolphthalein was used as the indicator in the titration and that the point of reference, i. e., the changing  $P_H$  of phenolphthalein, is about 9.

We were confronted in this experiment with a choice of criteria for our judgment as to when such a medium is sugar free. It seemed that the  $H^+$  ion concentration data yielded nothing of value here. And if we relied on the turn in the titratable acidity curves to indicate the sugar-free point, which organisms was to be regarded as yielding reliable data? There was no question that the decision rested on the true interpretation of the remarkable result with *B. welchii*. If the peak of the Welch titration curve were to be accepted as the sugar-free point, a much longer time would have to be allowed than is customary for freeing medium from sugar when the coagulable proteins are not removed.

We undertook to settle these points by comparing the acid production of *B. welchii* in meat peptone mash with that in some of the same medium from which the coagulated meat had been removed by filtration. This experiment was performed twice with essentially similar results, which are shown for one of the trials in chart 8.

The usual initial rise in  $H^+$  ion concentration and titer was observed irrespective of the presence of excess of meat. This confirmed our belief that the medium used in exper. 7 was deficient in sugar. The damping effect of the meat mash medium is a feature in the  $H^+$  ion concentration curves; a distinctly wider range of  $P_H$  values was recorded for the peptone broth medium.

The initial peak in the titration curve in the meat mash medium, followed by a secondary and higher peak as observed in exper. 7 was accentuated in exper. 8. The lack of any secondary rise in titer in the more lightly buffered peptone broth suggested that the secondary rise in titer in the meat mash medium was due to the admittedly weak proteolytic action of *B. welchii* on undigested protein. We have interpreted the first peak as the phase of sugar metabolism, the second, the phase of protein metabolism.

The experiments support our belief that a change in the direction of greater  $H^+$  ion concentration (lower  $P_H$  value) constitutes the best criterion of fermentation; titration indicates not only acidity but may indicate increased buffer capacity brought about by proteolysis and is therefore an unsafe guide in determining the point at which a given medium can be said to be free from sugar.

## REFERMENTATION TESTS

Most bacteriologists regard the sugar effectively removed from a medium when it fails to produce gas on refermentation with *Bact. coli*. It occurred to us that some information might be secured by cross fermentation tests between the three cultures here represented.

The samples removed from the flasks in exper 7 were utilized for the purpose, being first filtered through paper, and then tubed in the constricted fermentation tube with marble seal designed by one of us and sterilized in the autoclave. When the last of the samples in exper 7 were so collected all were inoculated and incubated at 37 C. for 4 days. Each tube was examined for contamination by subcultures of plain agar.

It was striking that none of the refermentation tests of the Welch broth gave gas. It must be admitted that the Welch bacillus itself failed to grow, unless we can regard the slight changes in reaction as indicative of growth. We note that these changes were in the direction of greater acidity above the heavy line in the table, which marks the peak of the titer in the Welch broth and in the direction of greater alkalinity below that line.

In the case of *Bact. coli* and *Bact. saccharolyte* all of the tests showed increased alkalinity—even in those samples removed before the peak of the Welch titer had been reached. Thus there is no evidence of a failure of *B. welchii* to remove all the fermentable sugar from the medium.

Contrasting the behavior of *Bact. coli* broth with *B. welchii* broth we note that growth occurred in all samples tested. In all cases but one, that of the 48 hour broth, *B. welchii* produced an increase in acidity and in all but the 24 hour and 48 hour samples, rather abundant gas. The data indicate the incomplete removal of sugar by *Bact. coli*. Yet the colon bacterium failed to produce gas in any of the samples and all became more alkaline, as might be expected from the fact that the peak of the acid titer was reached in the colon broth at about 24 hours. The same was true of the refermentation of the colon broth by *Bact. saccharolyte* except that in one instance a small amount of gas was produced.

The observations made for the colon broth apply in the main also to the *saccharolyte* broth except that no growth of *B. welchii* occurred in samples less than 48 hours old. Both acid and gas were produced by *B. welchii*, showing the incompleteness of sugar removal by *Bact. saccharolyte*. Yet this broth would be considered satisfactory from the standpoint of refermentation by *Bact. coli* and *Bact. saccharolyte*, neither of which produced acid or gas.

## FILTRATION EXPERIMENTS

If a clear "sugar-free" medium is desired after the use of *Bact. coli* or *Bact. saccharolyte* in its preparation, filtration through paper is not sufficient to give a satisfactory product; adsorbents, such as tale and diatomaceous earth, may, however, be used as clearing agents. While we have frequently had occasion to utilize adsorbents in clearing mediums, their investigation is not a part of the present communication. We were interested in comparing the filtering properties of cultures of *B. welchii* with those of *Bact. coli* and *Bact. saccharolyte* with respect to rapidity of filtration and clarity of product.

Exper. 9: The autoclaved cultures from exper. 3 were used. One hundred c c portions were taken from each flask after thorough agitation of the contents. The two portions of killed culture were then poured simultaneously into similar funnels containing the same size and type of filter paper. The filtrates were collected in graduated cones. The colon cultures passed through the paper more rapidly than did the culture of *B. welchii*, but the filtrate obtained from the latter was far superior to the colon filtrate as regards clarity.

Exper. 10: In this experiment the cultures were filtered without previous agitation. This time the Welch culture passed through the paper more rapidly than the colon culture. The quality of the filtrate favored the Welch product in clarity.

Exper. 11: Twenty-four hour broth cultures (400 c c each) of *B. coli* and *B. welchii* were autoclaved for 20 minutes at 15 pounds pressure. Immediately after the pressure was down and without agitation, 100 c c portions of each culture were removed and filtered simultaneously through one thickness of medium grade filter paper. The colon culture passed through the paper twice as rapidly as did the Welch culture, but the filtrate obtained was not nearly as clear as that obtained from *B. welchii*. Both filtrates were then refiltered; this time the Welch filtrate passed through the paper faster than the coli filtrate. The second filtrate of *B. welchii* was as clear as ordinary beef broth whereas the *Bact. coli* filtrate was still quite murky and required the use of a clearing agent to bring it to a parity with the Welch product. This experiment was repeated with identical results.

At this time a more serious consideration of the filtrability problem was conceived in order to detect any possible relation of filtrability to the stage of fermentation in which incubation was stopped. Thus the samples removed in exper. 7 were utilized as follows:

Exper. 12: Constant volumes (75 c c) of the unheated samples were filtered through as uniform as possible pieces of filter paper.

It is seen that the initial filtrability of *B. welchii* broth was low during the early stages of growth in meat peptone mash but increased as the culture became older, and especially after it passed the peak of the titration curve. The mucoid tenacity of this culture when young may account for the slowness of filtration at that stage of its growth.

The filtrability of *Bact. coli* broth was high and became better as the culture aged; that of *Bact. saccharolyte* was at a maximum and approximately equivalent to that of *B. coli* during the first 24 hours, after which it became progressively poorer.

The clarity of the products of filtration of *Bact. coli* and *Bact. saccharolyte* was at all times inferior to that of *B. welchii*.

We recognize the limitation of these observations to meat peptone mash; mediums with a lower protein content and particularly a lower coagulable albumin content might readily give different results.

#### SUMMARY

In this paper we propose the use of *B. welchii* in the preparation of "sugar-free" broth. This suggestion grows out of the well-known vigorous fermentative ability of this organism. It is suggested that the fermentation be conducted with culture medium to which all of the protein substances to be used in it have been added. No special means of providing anaerobiosis for the growth of *B. welchii* in such mediums are necessary other than inoculation before the medium is cool after sterilization, since the depth in quantities such as are ordinarily used provides an adequate exclusion of air. After growth starts the evolution of gas produced by fermentation automatically removes oxygen.

The fermentation should be followed by means of acidity determinations. Both on theoretical and practical grounds an increase in  $H^+$  concentration provides the best criterion of sugar splitting. When the protein buffer content of the medium is high, titratable acidity is an unsafe guide in determining when a given medium can be said to be free from fermentable sugar. In the present state of our knowledge of the use of both methods of acidity determination from which the acid curves may be plotted would seem superior to the present rule of thumb methods.

We have not been able to use chemical sugar tests of sufficient delicacy to detect traces of sugar in broth which can be demonstrated by cross fermentation tests. The results of cross fermentation tests, on the contrary, support the interpretation of the acidity tests proposed. Cross fermentation tests show that *B. welchii* is still able to produce acid and gas from mediums presumably exhausted by *Bact. coli* and *Bact. saccharolyte*. These organisms are not able to produce either acid or gas in mediums fermented out with *B. welchii*. This fact indicates the greater completeness of fermentation by *B. welchii* and

may actually point to the existence in broth of carbohydrates not fermented by the aerobes studied, possibly more complex sugars than the monosaccharides.

The time required for elimination of sugar by *B. welchii*, *Bact. coli*, and *Bact. saccharolyte* is approximately equal. But *saccharolyte* generally produces its maximum acidity in the shortest time—always less than 30 hours in the mediums studied. There is no choice among these organisms from this standpoint.

While the "sugar-free" broth produced by *B. welchii* filters through paper more slowly than that of *Bact. coli* and *Bact. saccharolyte* in the early stages of fermentation, its filtrability improves as the culture ages, and the product is at all times much clearer than that of *Bact. coli* and *Bact. saccharolyte* so that the use of adsorbents for clarification is unnecessary.

## COMPARISON OF FORMOL AND WASSERMANN REACTIONS IN DIAGNOSIS OF SYPHILIS

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In a recent paper Gaté and Papacostas<sup>1</sup> report that the addition of a small quantity of formalin to pooled syphilitic serum led to coagulation ("Gélification"), while no such reaction took place if the serums were from nonsyphilitic patients. The work was extended with individual serums, and at the same time controlled by parallel Wassermann tests. They found in a series of 400 comparative tests an agreement of 85% between the two reactions. To obtain the best possible results these authors then advocated the following method:

"To one cc of a clear serum two drops of commercial formalin is added. The mixture is then gently shaken and the tube plugged with cotton. The mixture is allowed to stand for 24 to 30 hours at room temperature and the results taken." They further found that inactivation of the serum was not a necessary factor, and that serum 48 hours old or even older gave identical results when compared with the same specimen freshly drawn, but they insist that the serums should not be contaminated. Incubation temperature does not seem to modify the reaction, and pooled positives always gelify in the presence of formalin. No explanation of the reaction is given. Pauzat,<sup>2</sup> however, found that from 57 comparative tests in which 11 were Wassermann positive, only 3 reacted by the formalin method, and from 46 negatives 6 gave a positive reaction by the new method. This author therefore doubts the diagnostic value of the formol reaction.

I have applied this reaction in a series of 500 comparative tests in which the Wassermann reaction was carried out by the icebox method with 3 different antigens, namely, syphilitic fetal liver, normal human and beef heart antigens. Of the 500 tests, 7 were designated  $\pm$  reactions because of some fixation in the presence of the syphilitic liver antigen. The remaining 493 tests were + + +, + + + + or entirely negative. In reading the results of the formol reaction a dis-

Received for publication May 27, 1921.

<sup>1</sup> Compt. rend. de la Soc. de Biol., 1920, 83, p. 1432.

<sup>2</sup> Ibid., 1920, 84, p. 503.

tinently increased viscosity was designated as +, marked viscosity as ++, and complete coagulation as +++. The serums were fresh and inactivated. Both Schering's and C. P. formalin were used in the determinations and in both acid and neutralized solutions. The incubation period varied from 24 to 48 hours and at temperatures of either icebox, room or 37 C. Some tubes were plugged with cotton, while others were tightly corked to prevent any loss by evaporation.

Table 1 gives the results of the series:

TABLE 1

Number of Serums Tested	Condition of Serums	Kind of Formalin Used	Incubation		No. of Wassermann Reactions		No. of Gelification Reactions		No. of Wassermanns Positive and Gelifications Negative	No. of Gelifications Positive and Wassermanns Negative	No. of Agreements
			Temperature	Time in Hours	Positive	Negative	Positive	Negative			
71	Fresh	Commercial	Room	26	24	47	18	53	18	12	6
59	Fresh	Comm. neutral	Room	24	22	37	20	39	12	10	10
29	Fresh	Commercial	Room	24	3	26	11	18	1	9	2
30	Fresh	C. P. acid	Room	24	4	26	6	24	3	5	1
45*	Inactivated	C. P. acid	Room	24	16	29	18	27	9	11	7
48	Inactivated	Commercial	Room	24	10	38	8	40	8	6	2
33	Inactivated	Commercial	Room	48	5	28	5	28	3	3	2
29	Inactivated	C. P. neutral	Room	24	8	21	4	25	5	1	3
19	Inactivated	Commercial	37 C.	30	4	15	4	15	3	3	1
30	Inactivated	C. P. neutral	Icebox	30	6	24	2	28	4	0	2
49	Inactivated	Commercial	Room	25	7	42	11	38	4	8	3
58	Inactivated	C. P.	Room	48	13	45	17	41	5	9	8
Total 560	.....	.....	.....	.....	122	..	124	..	75	77	47
Percentages.....	.....	.....	.....	.....	24.40	..	24.80	..	15	15.4	9.4

Seven Wassermanns were  $\pm$  with syphilitic fetal liver.

From this comparative summary it is seen that the total number of agreement in both tests varies considerably depending in part on variations in the formol-test technic. Of the final number of positives obtained by the new method only 37.09% were positive by the Wassermann test, the figure being markedly lower than that obtained by Gaté and his associate and slightly higher than that of Pauzat which was 27.27%. Furthermore, 44 positive tests by the formol method



were of the + type, and of these only 13 were Wassermann positives, leaving the rest unaccounted for. Four formol + positives were known negatives.

#### CONCLUSIONS

Of the total number of positive reactions obtained by the formol reaction of Gaté and Papacostas, only 37.09% agreed with the positive results obtained by the Wassermann method.

A large number of formol positives (44 or 8.8% of total) were of the + type, and of these 13 (or 29.54%) were positive by the Wassermann method. These weakly positive reactions tend to induce confusion, as it is often difficult to interpret these reactions.

The reaction as it stands is of no diagnostic value because of its failure to react in clinically and serologically clear cut cases of syphilis, and the occurrence of positive reaction in the absence of the disease.

# THE THERMAL DEATH POINT OF THE SPORES OF BACILLUS BOTULINUS IN CANNED FOODS

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I reported <sup>1</sup> the results of an investigation of the resistance of the spores of *B. botulinus* <sup>2</sup> to heat and the effects of several variable conditions which influence their death. It was shown that the thermal death point is markedly influenced by the hydrogen-ion concentration. It was also shown that the destruction of the spore is a gradual process, not an instantaneous killing; that is, the spores are progressively injured and finally destroyed. It was demonstrated that spores of different strains vary considerably in their heat resistance; that the resistance is influenced by the age of the spores; that young spores about 1 month old have the highest thermal resistance; that increasing the concentration of sodium chloride decreases the resistance; and that the larger the number of spores the higher the temperature and longer the period of exposure required to kill them. The relative importance of these factors was also shown.

On the basis of these facts I proceeded to a study of the heat resistance of the spores of *B. botulinus* in commercially canned foods. Thirty-six varieties of standard brands of foods on the American market were tested.

## TECHNIC

The can liquor was removed with aseptic precautions. In cans in which there was no free liquor the juice was expressed through sterile gauze into a sterile container. The spore suspension was prepared by growing *B. botulinus* in sheep's brain medium for one month and straining through sterile gauze. Nine parts of the food fluid to be tested was mixed with one part of the spore suspension, and 1 cc of this mixture was pipetted into a tube, 10 mm. inside diameter, 12 mm. outside diameter and 30 cm. long. A series of such tubes were prepared. Special tubes were used, care being required in their manufacture and selection to insure a uniform quality of glass and a uniform thickness of wall to obviate experimental errors arising from the influence of these factors on the rate of heat penetration. The change

Received for publication May 31, 1921.

<sup>1</sup> Weiss, H.: The Heat Resistance of Spores with Special Reference to the Spores of *B. botulinus*, *Jour. Infect. Dis.*, 28, p. 70.

<sup>2</sup> Committee of Soc. Am. Bact. (*Jour. Bacteriol.*, 1920, 5, p. 222) recommends that this organism be called *Clostridium botulinum*.

In *Hygienic Laboratory Bull.* No. 121, Sept., 1920, E. M. A. Enlows, Jensen classifies this organism as *Botulobacillus botulinus*.

in the  $P_H$  value due to the glass was determined and was found to be negligible under our experimental conditions. The tubes were heated in a Bunsen flame to within 2 cm. of the surface of the fluid in order to kill all spores which might have been deposited on the glass. When these had cooled, they were sealed in the flame submerged to a depth of 12 cm. in a DeKhotinsky oil bath and exposed to the action of heat. A series of tubes was prepared in this manner for exposure at a given temperature, and at certain intervals a tube was removed, opened, and 10 cc of freshly heated glucose agar added, care being taken to mix thoroughly the spore suspension throughout the agar. The tubes were then incubated at 37.5 C. for at least 3 months. The long period of incubation was necessary in order to determine delayed germination of injured spores.<sup>1</sup>

The experiment was planned to determine the thermal death point of the spores of *B. botulinus* under laboratory conditions arranged to approximate as nearly as practicable the conditions in the can. The food juice was tested immediately after its removal from the can, its hydrogen-ion concentration determined and a note made of the consistency of the liquor obtained. This consistency varied from a colored or almost colorless watery solution in some products, such as Brussels sprouts, string beans, peas, asparagus, etc., to an opaque, heavy, almost gelatinous mixture in others, such as succotash, pork and beans, chili con carne, etc. The influence of the physical constitution of the food juice will be indicated later.

One strain of *B. botulinus* (our laboratory strain 15) was used in all the exposures. It was originally isolated by Edmondson from asparagus salad, which had caused the death of 4 persons at Boise, Idaho, in January, 1919. This strain was chosen from 16 because it produces spores of a higher resistance than any other strain of unquestionable origin; its identity is certain; it is an active gas producer on glucose mediums and produces a toxin of high virulence.

It has been shown<sup>1</sup> that the thermal resistance of a spore suspension varies with the age of the spore, the greatest resistance being found to exist when the spore is about 1 month old. In order to keep this factor constant spores of this age were employed.

The degree of heat and time of exposure necessary to accomplish sterility vary with the number of spores present in the suspension. The greater the number, the more heat is required. The initial spore suspension as grown contains approximately 15,000,000 spores per c.c. One part of this suspension strained through sterile gauze was diluted with 9 parts of the food liquor. After dilution each tube contained

1,500,000 spores in 1 c.c. of volume. I have chosen 1,500,000 spores per c.c. as a standard concentration, believing that that is far greater than the number of spores that can possibly be present as a contamination in any canned product.

#### RESULTS

The results indicate that the size of the container being constant, there are at least two primary factors that determine the length of exposure and degree of heat required to accomplish sterility: (1) the hydrogen-ion concentration, and (2) the physical character or consistency of the food.

I have shown<sup>1</sup> that the spores of *B. botulinus* show their greatest thermal resistance at the point of neutrality or very near that point when pure acids and alkali are used, and that the thermal resistance rapidly diminishes as the hydrogen-ion concentration increases or decreases from that point. The results with the juices of canned food products substantiate this conclusion (see diagram).

Analysis of the results show that the spores of *B. botulinus* are killed in all food juices (such as the 11 fruit products given in the table) having a  $p_H$  value between 2.1 and 3.85 in 50 minutes or less at 100 C. The greater number of these fruit juices require only 30 minutes or less. Less acid foods having a  $p_H$  of 4.22 and 4.4 require 60 to 90 minutes at the same temperature. Beets, asparagus, wax beans, peas, squash and sweet potatoes having  $p_H$  values of 5.13 to 5.36 require an exposure of 90 to 120 minutes at 100 C. to kill the spores of *B. botulinus*. Pork and beans, red kidney beans, lima beans, chile con carne, succotash and sweet corn with  $p_H$  values of 5.69 to 6.21 require 150 to 180 minutes at 100 C. to accomplish the same result.

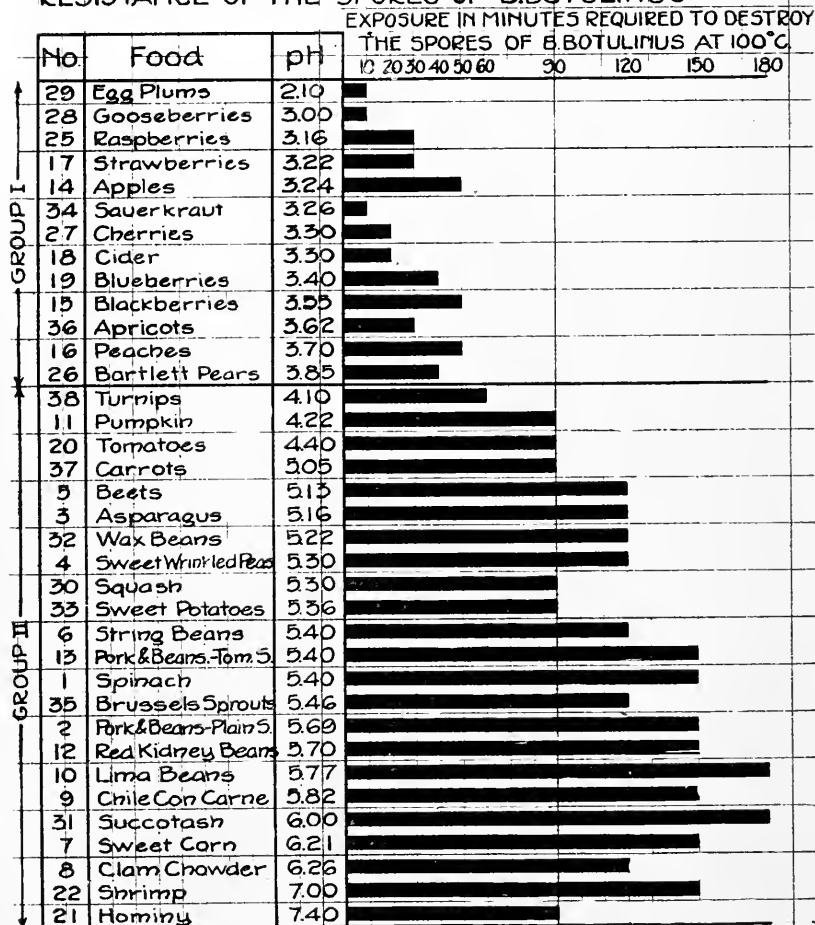
Other factors being equal, foods having an alkaline reaction as well as those having an acid reaction, require a shorter period of exposure to kill the spores of *B. botulinus* than food which has a neutral reaction. For example, food number 21 (hominy) with a  $p_H$  value of 7.4 required only 90 minutes at 100 C., while food number 22 (shrimp) with a  $p_H$  value of 7.00 required 150 minutes or almost twice that period of exposure. The results are shown graphically in the accompanying tabular diagram (see diagram).

Another fact that is apparent is that all the fruits and fruit products tested form a group showing the highest acidity. The  $p_H$  values of the products in this group fall between 2.1 and 3.85, and the maximum period of exposure required at 100 C. is 50 minutes. The second group

TABLE 1  
THERMAL DEATH POINT OF THE SPORES OF *B. BOTULINUS* IN CANNED GOODS

[illegible]

TABULAR DIAGRAM SHOWING THE EFFECT OF THE  
pH VALUE OF VARIOUS CANNED FOODS ON THE THERMAL  
RESISTANCE OF THE SPORES OF B.BOTULINUS —



with  $p_H$  values ranging from 4.1 to 7.4 requires an exposure of 90 minutes to 180 minutes at the same temperature with the single exception of turnips which require 60 minutes, their  $p_H$  value being 4.0, thus most nearly approaching the hydrogen-ion concentration in the fruit group. This second group includes all the vegetable products to the complete exclusion of all the fruit products.

It is evident from the diagram that in the first group a number of products of almost equal  $p_H$  value show differences of 10 and 20 minutes in the exposure required to kill the spores. This difference is apparently due in most cases to the concentration of syrup present in the product, the greater the concentration of syrup the longer the period of exposure required.

In the second group, 3 products—string beans, pork and beans in tomato sauce and spinach—with similar  $p_H$  value, show a variation of 30 minutes in the exposure required at 100 C. to kill the spores. This is apparently due to the consistency of the foods in question. Of these 3 foods having a  $p_H$  value of 5.40, string beans, which have a loose consistency and are completely bathed in liquor, require 120 minutes at 100 C., while pork and beans in tomato sauce and spinach require 30 minutes longer, due to the fact that these products are of a heavier or thicker consistency. Similarly, the liquor of Brussels sprouts with a  $p_H$  value of 5.46 requires an exposure of 120 minutes at 100 C., while the liquor of pork and beans in plain sauce and red kidney beans, which have only a slightly higher  $p_H$  value (5.69 and 5.70), requires 150 minutes at the same temperature. Succotash, sweet corn and clam chowder, having  $p_H$  values of 6.00, 6.21 and 6.26, are more fluid in the order named, and as a result require 180, 150 and 120 minutes exposure, respectively.

Another important factor in the sterilization of canned foods is the time required for the heat to penetrate to the center of the can. This phase has been fully treated by Bigelow, Bohart, Richardson and Ball.<sup>2</sup> The effective temperature and periods of heating given in this paper are exclusive of the period of penetration which varies with the particular food and the size of the can. For practical purposes the period of exposure required for any food product will be expressed by the period given plus a factor depending on the size of the can, the specific coefficient of heat penetration, the retort technic, etc.

<sup>2</sup> Bull. 16-L, Research Laboratory, National Canners Association, Washington, D. C.

## SUMMARY

The thermal death point of the spores of *B. botulinus* in the juices of 36 varieties of canned food on the American market has been determined.

The thermal death point varies with the hydrogen-ion concentration of the particular food in question. The more acid foods, such as canned fruits, require a maximum of 50 minutes at 100 C., 30 minutes at 105 C. and 15 minutes at 110 C.; a majority of this group require much shorter exposures at the temperatures given. Thus, 8 of the 11 food products in this first group require 30 minutes or less at 100 C. The vegetable products, which are less acid and more nearly approach the neutral reaction, require from 90 to 180 minutes' exposure at 100 C., 30 to 70 minutes' exposure at 105 C., and 10 to 20 minutes' exposure at 110 C.

The thermal death point also depends on the consistency of the particular food, the more fluid products requiring a shorter period of exposure at a given temperature than the less fluid ones.

The thermal death point is also influenced by the presence and concentration of syrup. The heavier the syrup, the longer the period of exposure required at any one temperature.

The temperatures and times of exposure given in this paper must not be directly applied to practical canning, for these factors vary not only with the nature and the  $p_H$  value of the food in question, but also with the size of the can, the size and compactness of the cook and the retort technic. Furthermore; a factor of safety should be added to insure sterilization under practical commercial conditions.



# THE EFFECTS OF DIET ON THE INTESTINAL FLORA

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It has long been recognized that certain pathologic conditions in man are associated with an intestinal flora that is markedly putrefactive in type. Headaches, skin disorders, digestive disturbances, nonalcoholic cirrhosis, nervous abnormalities and cardio-vascular-renal disease have been attributed with more or less reason to the effects of excessive intestinal putrefaction. Metchnikoff<sup>1</sup> believed that premature senescence was the result of this bacterial activity, and the term "auto-intoxication" became almost a household word as the result of his theories. Many of Metchnikoff's assumptions were not founded on definitely established facts, and it has since been shown that in many of the cases of so-called "auto-intoxication" the abnormal symptoms were really the result of chronic infections in the digestive tract or elsewhere. When these foci of infection were removed the symptoms disappeared. In other cases the symptoms have been shown to be the result of nervous reflexes. After eliminating these cases, however, there are still many instances of acute and chronic conditions in man in which no focal infections have been found and in which there is definite evidence of an intoxication of intestinal origin.

It is generally assumed that these toxemias are due to the absorption of poisonous substances from the lumen of the intestinal tract and that these poisonous materials are formed as the result of the action of proteolytic bacteria on proteins or their split products. The presence of putrefactive products and of certain toxic amines in intestinal contents has long been known. Furthermore, it is well known that members of the colon group of bacteria have the ability in vitro of forming indol, skatol, phenol, hydrogen sulphide and certain toxic amines, such as histamine and tyramine. We know that some of these products are absorbed and excreted by the kidneys without any apparent ill effects. The evidence in regard to the absorption of toxic amines

Received for publication May 31, 1921.

\* Part of the work was carried on in the Department of Pathology and Bacteriology, The University of Mississippi.

<sup>1</sup> The Prolongation of Life.

is not so clear, nor is it known to what extent the liver functions as a detoxicating organ when unusually large amounts of these substances are absorbed.

In certain acute conditions, such as intestinal obstruction, diarrheas and digestive disturbances in children, there is a considerable amount of evidence that these poisonous substances are absorbed to an unusual degree, and that the toxemia is the result of the increased absorption of these bacterial products. Dragstedt<sup>2</sup> and his coworkers have shown that in acute intestinal obstruction the presence of bacteria in the lumen of the intestine is necessary for the production of these toxic substances and that in their absence no poisonous materials are formed. In later work<sup>3</sup> it was shown that the toxemia incident to acute obstruction is uniformly associated with a proteolytic intestinal flora, irrespective of the nature of the flora before the obstruction was produced. In animals with an aciduric flora, experimental intestinal obstruction led to a very slowly developing toxemia as compared with animals in which a proteolytic flora was present at the time of operation. In diarrheal conditions it has been observed that the toxemia is profound when the diarrhea is of the putrefactive type, whereas in the fermentative type there may be little evidence of toxemia. Certain digestive disorders in children have been shown to be associated with a putrefactive intestinal flora. Morris, Porter and Meyer<sup>4</sup> have described such cases in which the flora was controlled by diet. These workers found that the return of these children to normal health was coincident with a regression of the intestinal flora toward predominantly fermentative types. The improvement in each case began at a time when it was possible to show that the intestinal flora had altered in type from putrefactive to fermentative.

Attempts to devise methods of preventing this putrefaction have been persistent and varied. Vaccines, intestinal antiseptics, removal of the colon, and implantation of antagonistic organisms have been tried with slight success. One popular procedure has been the use of lactic acid milks, particularly milk fermented by *B. bulgaricus*. The original belief was that this organism could be implanted in the intestinal tract and that it formed enough acid to inhibit the growth of the proteolytic types. As a whole, the use of this method has been disappointing and

<sup>2</sup> Jour. Exper. Med., 1917, 25, p. 421; 1919, 30, p. 109; Am. Jour. Physiol., 1918, 46, p. 366; Jour. Exper. Med. 1918, 27, p. 359.

<sup>3</sup> Cannon, P. R.; Dragstedt, L. R., and Dragstedt, C. A.: Jour. Infect. Dis., 1920, 27, p. 139.

<sup>4</sup> Jour. Infect. Dis., 1919, 25, p. 349.

within the last few years observations have shown that it is impossible to implant *B. bulgaricus* in the intestinal tract, since the organism is apparently not an inhabitant of the normal intestine and cannot adapt itself to its new environment.

The other method of lessening intestinal putrefaction is the substitution of carbohydrate food for protein, and within recent years many workers have clearly shown that under normal physiologic conditions the chemical character of the food ingested is the fundamental factor controlling the bacteria of the intestinal tract. This conception was advanced by Hirschler,<sup>5</sup> in 1886, when he found that in the case of dogs fed cane sugar, potatoes, glycerol and dextrin with meat, the feces contained indol and phenol in smaller amounts than in those fed meat alone. Winternitz,<sup>6</sup> a few years later, concluded that the feeding of milk tends to inhibit protein putrefaction and retards the formation of the protein split products. He attributed this influence to the lactose and believed that it was independent of the effect of the lactic acid. Through milk feeding, he claims, there is a decrease in the ethereal sulphates of the urine and a lessening of the last protein split products "worthless for the host and perhaps harmful."

Herter and Kendall<sup>7</sup> were the first (1908) to show clearly the effects of diet on the bacteria of the intestinal tract. They found that the intestinal flora of cats and monkeys was rapidly altered when a diet of meat and eggs was followed by one of milk and dextrose, there being a substitution of an aciduric nonproteolyzing type for one that was strongly proteolytic. In the same year Jungano<sup>8</sup> showed that the intestinal flora of white rats was changed when a meat diet was fed, *B. coli* then becoming the predominant organism although it had been absent or nearly so with the ordinary diet. De Gasperi<sup>9</sup> obtained about the same results as Jungano, finding that with an ordinary diet of wheat and bread the aciduric organisms were more prominent and with a meat diet the proteolytic bacteria appeared in great numbers.

Kendall has shown that in vitro proteolytic organisms are less active in the presence of carbohydrates, fermentation instead of putrefaction resulting. It is now a well-known physiologic fact that body cells and bacteria will obtain their carbon requirements from carbo-

<sup>5</sup> Ztschr. Physiol. Chem., 1886, 10, p. 306.

<sup>6</sup> Ztschr. f. Physiol. Chem., 1892, 16, p. 460.

<sup>7</sup> Jour. Biol. Chem., 1908, 5, p. 293.

<sup>8</sup> Compt. rend. Soc. de Biol., 1909, 66, pp. 112, 122.

<sup>9</sup> Centralbl. f. Bakteriol., I, O., 1911, 62, p. 519.

hydrates in preference to proteins and amino acids. It is only in the absence of carbohydrates that proteins are torn to pieces to supply energy. With this idea as a basis, several workers since 1908 have studied the effects of carbohydrate feeding on the intestinal flora. Sittler,<sup>10</sup> in that year, found that when lactose was fed to children on a cow's milk diet, *B. bifidus* became the predominant organism, and he concluded that the presence of *B. bifidus* in the intestines of breast-fed infants was due to the large amount of lactose in human milk. He showed that sucrose and levulose are unfavorable for the development of *B. bifidus*, while *B. acidophilus* becomes the predominating organism in infants fed malt soup.

Of the particular carbohydrates tested since that time, lactose and dextrin appear to be by far the most effective in transforming the intestinal flora. Distaso and Schiller<sup>11</sup> demonstrated with white rats that by feeding lactose or dextrin with bread and meat the intestinal flora could be changed into one consisting almost entirely of *B. bifidus*. They believed that these sugars arrived in the lower part of the intestines almost intact because of the lack of proper digestive ferments. When the sugars reached the region of bacterial activity the bacteria fermented them, and soon fermentative organisms controlled the field. Rettger and Horton,<sup>12</sup> in the same year, showed that when the diet of white rats was changed to one containing starch, lard, protein-free milk and a pure protein, the intestinal flora became much simpler and gram-positive organisms constituted from 85 to 100 % of the field as against 35 to 50 % with the ordinary mixed diet. Two organisms—*B. acidophilus* and *B. bifidus*—were frequently present to the exclusion of all other types. *B. coli* was also reduced in numbers in the special diet rats. Hull and Rettger<sup>13</sup> continued this work by studying the effects of various carbohydrates when fed to white rats with bread and vegetables. They used lactose, maltose, sucrose, dextrose, levulose, galactose, and dextrin. In the case of lactose *B. bifidus* soon became the predominating organism and often with few other bacteria accompanying it. The other carbohydrates gave negative results.

In a later publication<sup>14</sup> the same authors gave the conclusion of several years' study, namely, that a diet high in lactose was the most

<sup>10</sup> Ibid., I, O., 1908, 67, p. 14.

<sup>11</sup> Compt. rend. Soc. de Biol., 1914, 76, p. 243.

<sup>12</sup> Centralbl. f. Bakteriol., I, O., 1914, 73, p. 362.

<sup>13</sup> Ibid., 1914, 75, p. 219.

<sup>14</sup> Jour. Bacteriol., 1917, 2, p. 47.

effective in establishing an aciduric intestinal flora. Lactose in amounts of 1 to 2 gm. or more per day with a mixed diet led to a complete simplification of the flora in white rats. Milk and mixed grains tended to increase the number of aciduric bacteria, whereas meat increased the number of indol-producing bacteria. They also found that a high lactose diet in man led to the development of aciduric bacteria in large numbers. Torrey,<sup>15</sup> working with dogs, came to essentially the same conclusion as Rettger et al. Lactose and dextrin, when fed with a meat and rice diet, led to a marked replacement of proteolytic organisms by aciduric ones. Saccharose, maltose, and dextrose caused no pronounced change, although saccharose in large amounts caused a rise in the numbers of obligate fermentative bacteria. Starchy food led to a simplification of the intestinal flora and the development of *B. acidophilus* to a considerable extent. Vegetable proteins exerted a marked antiputrefactive tendency in certain cases. Mammalian tissues seemed to be the only ones which markedly encouraged the growth of putrefactive organisms in the intestines. Fish and casein did not encourage the development of putrefactive bacteria to the same extent as mammalian tissue. Fats exerted little effect on the flora.

The work here reported is based upon the idea that diet is the fundamental factor controlling the activities of the bacteria of the intestinal tract. The effort throughout the investigation has been to get a quantitative idea of the relative abundance of the different organisms, and with this end in view, particular attention has been paid to methods for the estimation of the numbers of bacteria of each type and the effects of diet on their presence.

#### METHODS

White rats have been used in this work because of the ease in handling, the small expense of feeding and because a large number of individuals can be used, thus ruling out a large part of the error due to individual idiosyncrasy. The feces have always been collected as far as possible in the manner described by Hull and Rettger. The rats were held by the tail and rubbed on the back above the base of the tail. In most cases the feces can be collected into tubes of sterile water or on clean paper and are always fresh. These are then emulsified in 10 cc of sterile water and dilutions of 1:1000, 1:10,000 and 1:100,000 made. No effort has been made to weight out the feces as they vary so much in water content and residue in the various diets that only a fictitious accuracy would be attained. The effort throughout has been to determine the relative numbers of the different types rather than the numbers per unit weight of feces.

<sup>15</sup> Jour. Med. Research, 1919, 39, p. 15.

Torrey has shown that fecal bacteria in general may be conveniently grouped in accordance with their metabolic tendencies. According to this classification, there are two main groups composed of organisms which are predominantly fermentative or putrefactive in their tendencies. Intermediate between these are the members of the *B. coli* group which are either fermentative or putrefactive, depending on the nature of the food at their disposal. Members of this group are the predominant types of the aerobic intestinal bacteria in man and many of the lower animals, constituting about 60% of the viable bacteria of the feces, according to Kendall. In man at least their activities are usually more proteolytic than fermentative. *B. acidophilus*, on the other hand, is relatively abundant in children and is the predominant organism of the fermentative or aciduric group. Different foods cause a variation in the relative numbers of these two types, and this fact has been made the basis of the cultural work. By comparing the numbers of *B. coli* with those of *B. acidophilus* from the same fecal emulsion, a quantitative relationship can be shown. This relationship is expressed as the colon-acidophilus ratio or the C-A ratio. The following procedure has been followed in determining this ratio: The appropriate dilutions are made and carefully mixed and 1 cc portions added to sterile Petri dishes, using the same pipet in each case. The rule has been to make all platings in duplicate. To one set of plates the Ayers and Rupp<sup>16</sup> medium is added; to the other the beef liver glucose agar of Torrey.<sup>17</sup> The plates are incubated for 48 hours, and then the most favorable dilution for quantitative purposes is selected and the same dilution in the other medium is used with it. By using mediums adapted to these two types of organisms, other types are ruled out to a certain extent, and a greater accuracy is thereby attained. All typical red colonies in the Ayers and Rupp medium are considered *B. coli* and all the small fluffy colonies resembling a fleck of cotton in the Torrey medium are considered *B. acidophilus*. These mediums are also prepared in small flasks and so the pouring of many plates is an easy matter.

The percentage of aerobes producing hydrogen sulphide in any fecal emulsion may be determined in a similar manner by using the acetate agar in plates. This medium gives a more accurate count than acetate broth and is as convenient to use. For finding the proportion of spore forming anaerobes, it is of great value. This is determined as follows: A tight plug of sterile absorbent cotton is pushed to the bottom of the tube of fecal emulsion, thus separating the solid particles from the bacteria. The supernatant fluid is divided into two portions, one of which is heated at 80 C. for 20 minutes. Dilutions of each portion are then made and plated in acetate agar, the unheated one aerobically and the heated portion by the Krumwiede-Pratt method. At the end of 36 hours both sets of plates are counted, and the proportion of spores to total viable aerobes in the same emulsion is found. *B. welchii* forms brown colonies in this medium and a good idea of the relative abundance of spores of this organism may be readily determined in this way.

#### STAINS OF THE FECAL EMULSIONS

Considerable information may be obtained from gram stains of the fecal emulsion. The study of smears in more than 50 instances in which the C-A ratio was 1.99 showed the uniform presence of from

<sup>16</sup> Jour. Bacteriol., 1918, 3, p. 433.

<sup>17</sup> Ibid., 1917, 2, p. 435.

75 % to 99 % of slender gram-positive bacilli. In a great many cases in which the animals were on a diet of bread, milk and lactose, the fecal smears showed practically 100 % of slender gram-positive bacilli in bunches and palisade arrangement. On the other hand, if the flora is proteolytic in type, there is a great preponderance of gram-negative bacilli, spirilla and large gram-positive spore-forming bacilli.

#### MEDIUMS

The following mediums have been used and incubation at 37 C. has been the rule in all cases.

1. *Ayers and Rupp Agar*.—This medium is of distinct value in determining the numbers of *B. coli* and in that way ascertaining the effects of the diet on their relative abundance. The fact that the readings are made at the end of 48 hours makes this medium valuable in conjunction with Torrey's beef-liver agar. The reliability of this medium in determining the presence of *B. coli* is shown by the fact that of 152 colonies transferred to peptone broth, 149 formed indol, thus showing their proteolytic powers.

2. *Beef Liver Glucose Agar*.—I have found this solid plate method well adapted for the quantitative estimation of *B. acidophilus*. With an initial reaction of  $P_H$  5.7 to 6.0 the characteristic fluffy colonies are well developed at the end of 48 hours and are easily counted.

3. *Lead Acetate Agar*.—This medium has been useful in getting a quantitative idea of the presence of hydrogen-sulphide formers, both aerobic and anaerobic. This is a 2% agar prepared from beef infusion, containing 3% of peptone and 0.5% of NaCl. The reaction was  $P_H$  7.4 to 7.8. Armour's peptone has been the best of the peptones tried and has been used throughout the experiments. Just before the plates are poured, 2% of sterile 10% lead acetate in distilled water is added to the medium, which has been cooled to about 50 C. and thoroughly mixed. This medium is prepared in small flasks (250 cc), which facilitates the pouring of a large number of plates at one time. After the medium in the plates is hardened, about 15 cc of sterile 3% agar in water is poured over the surface. This prevents the growth of surface spreaders and also leads to a slight reduction in oxygen tension which apparently favors the production of hydrogen sulphide. In the plates the hydrogen sulphide producing organisms appear as dark brown colonies, in some instances with a brown zone surrounding them. By comparing the number of these colonies with the total number of colonies on the plate, the relative abundance of the aerobic hydrogen-sulphide-producing organisms may be ascertained. A great variety of organisms develop in this medium, including *B. acidophilus*, and apparently the total number of viable aerobic bacteria in any particular fecal emulsion may be quite accurately determined. In the same manner the relative proportion of spore-forming anaerobes may be found, using the Krumwiede and Pratt method of pouring the medium into the cover of the Petri dish, placing the bottom on it and sealing the edge with sterile paraffin.

4. *Sheep Brain Medium*.—This medium is prepared according to the method of Hall<sup>18</sup> and is useful in determining the presence of putrefactive spore-forming anaerobes. These forms cause a distinct blackening of the medium,

<sup>18</sup> Jour. Infect. Dis., 1920, 27, p. 579.

as *B. sporogenes* for example, within 24 hours. By heating any particular fecal emulsion at 80 C. for 20 minutes, making dilutions in sterile water and inoculating 1 cc portions into tubes of the sheep brain, the presence and relative abundance of putrefactive types may be ascertained.

#### COLON-ACIDOPHILUS RATIO

The object of this work was to test out the methods described to determine the quantitative relationships of the two groups under the influence of certain diets. Rats were fed a stock diet of oats and carrots, ground beef, American cheese, meat and dextrin, and bread, milk and lactose. In addition, the effects of a diet high in lactose, dextrin, and vegetable protein were tested on three adults.

*Oats and Carrots.*—Rats on this stock diet for 2 weeks showed a large proportion of slender gram-positive bacilli in stains from the fecal emulsions. The colon-acidophilus ratio, the average of 1 plating from 4 rats, was 1:99.

*Ground Beef.*—The C-A ratio of rats on a high animal protein diet consisting almost exclusively of ground beef was 80:20. This figure is the average of 35 platings from 23 different animals which had been on the diet from 5 to 30 days. In older animals and in those that have been on the meat diet longer the C-A ratio rises, in many cases being 99:1. The stains from the fecal emulsions show a great preponderance of gram-negative organisms, the emulsions as a rule are foul smelling and give a much stronger indol reaction than do those of rats on either the stock diet of oats and carrots or bread, milk and lactose.

*American Cheese.*—Four rats were put on a bread, milk and lactose diet for 2 weeks. White bread was soaked in whole milk and mixed with lactose in the proportion of approximately 2 parts of bread to 1 of lactose. Their average C-A ratio at the end of this time, the result of 2 sets of platings, was 15:85. The rats were then fed only American cheese, with a small amount of carrot and cabbage mixture. At the end of 4 days the C-A ratio was 65:35, after 2 weeks 85:15, and at the end of 1 month 90:10. The diet was then changed, white bread and milk being substituted for the cheese. At the end of 1 week the C-A ratio was 6:94. These figures indicate that the relative abundance of proteolytic and aciduric organisms can be altered markedly in proportion to the presence of an abundant protein or carbohydrate diet.

*Dextrin.*—Torrey found dextrin even more effective than lactose in bringing about the establishment of an aciduric flora in dogs, the fecal flora being dominated by *B. acidophilus*. Hull and Rettger with white rats found dextrin effective in only a few instances, and in these the flora was not markedly simplified nor did *B. acidophilus* appear in great prominence. In later studies, however, Cheplin and Rettger<sup>19</sup> found dextrin as effective as lactose in stimulating the proliferation of *B. acidophilus* in white rats. My experience has been the same, dextrin leading to a marked simplification of the intestinal flora. Four rats were fed only ground beef, carrots and cabbage leaves for 3 weeks. Their average C-A ratio at the end of this time was 91:9. Dextrin and ground beef, 3 parts of dextrin to 7 parts of meat, were then fed. Four days later the average C-A ratio was 2:98, *B. acidophilus* having completely gained the ascendancy. The feces were light yellow in color with the dextrin diet.

<sup>19</sup> Abstr. of Bacteriol., 1920, 1, p. 8.



whereas they were black with the full meat diet, and the indol reaction in the former case was faint or absent. Streptococci were also increased in numbers in this diet, and the hydrogen-sulphide producing bacteria were practically eliminated.

*Lactose*.—This sugar, when added to a diet of bread and milk in the general proportions given, caused a striking change in the character and flora of the feces. Here, too, the feces were softer and a lighter yellow than those of the meat eaters; there was less odor, and the indol reaction with the fecal emulsion was slight and in many instances negative. Culturally the C-A ratio the average of 29 platings from 18 different rats that had been on this diet from 5 to 30 days was 1:99. The stains from the fecal emulsions showed a great preponderance of slender gram-positive bacilli.

#### EFFECT OF LACTOSE ON MAN

The effect of a diet of milk, toast and lactose was determined in the case of 2 adults. For a period of 10 days each person consumed one half pound of lactose daily in addition to the regular diet of milk and toast. On an average 800 gm. of bread (toasted) and 1 liter of whole milk was consumed daily by each. Little butter was used, not more than 10 gm. per day. H-2 also consumed 2 liters of sour milk daily during the 3d, 4th and 5th days of the experiment, and then this was discontinued. The lactose was suspended in milk or water and a little vanilla added to make it more palatable. The fecal specimens were obtained after epsom salts had been given, and presumably represented a somewhat higher level of the intestinal tract than if this had not been done. Dilutions were immediately made and plated in plain infusion agar, beef-liver agar, and Ayers and Rupp agar. The large amounts of lactose caused considerable abdominal distention at first, but during the last 3 days of the experiment this was not so noticeable.

H-1: At the beginning of the experiment the flora of this person, as shown by cultural tests, was typical of the average adult on a mixed diet. *B. coli* was the predominant organism, and stains showed a field which was composed predominantly of gram-negative bacilli with a few plump gram-positive bacilli and diplococci. At the end of 3 days there was a slight increase in the number of gram-positive bacilli and diplococci. At the end of 6 days, *B. coli* was still the predominant organism culturally but the slides showed many slender gram-positive beaded bacilli, singly and in filaments. At the end of 10 days about 50% of the organisms in the smears were these slender gram-positive beaded bacilli. The cultural tests were unsuccessful, so it is uncertain whether these were *B. acidophilus* or *B. bifidus*.

H-2: The flora of this person at the beginning of the experiment was similar to that of H-1, gram-negative bacilli being in predominance both culturally and in the stained smears. At the end of 3 days there was a slight increase in the number of slender gram-positive bacilli and diplococci. Two liters of sour milk were consumed daily during the next 3 days, and at the

end of this period about 59% of the organisms in the stains were gram-positive diplococci. Culturally about 95% were streptococci, presumably *Streptococcus lacticus*. The sour milk feeding was then discontinued, and at the end of the 10th day of the experiment the streptococci were practically gone. At this time about 50% of the organisms in the stained smears were slender gram-positive beaded bacilli. *B. acidophilus* appeared on the beef-liver plates, although here *B. coli* was in predominance. The disappearance of the streptococci after discontinuing the ingestion of the sour milk illustrates the difficulty of implanting foreign organisms in the intestinal tract.

H-3: The effects of a diet relatively high in vegetable protein were determined in an adult. This man consumed during a period of 10 days 29 meals, each consisting of 100 gm. of "black-eyed peas," 400 gm. of white bread (toasted), 10 gm. of butter and a small amount of lettuce. This furnished approximately 2,700 calories daily and was considered adequate for a student leading a sedentary life. Before the diet was begun the normal flora was determined by plating in acetate agar aerobically and anaerobically, beef-liver agar and Ayers and Rupp medium. In each case the feces were collected after the administration of epsom salts.

At the beginning of the experiment the fecal emulsions were typical of an adult on a fairly high animal protein diet. The stains showed a great predominance of gram-negative bacilli and many large plump gram-positive bacilli. Aerobic hydrogen-sulphide-producing organisms were not prominent, but anaerobic spore-forming organisms were in great abundance and consisted almost exclusively of *B. welchii*. (Thirty-seven separate brown colonies from the anaerobic plates were picked to litmus milk and incubated anaerobically. All gave a typical "stormy fermentation" with butyric acid odor. These were transferred to dextrose broth and dextrose agar and found to be anaerobic nonmotile bacilli. Furthermore, the 3-day milk cultures were heated at 80 C. for 20 minutes and then 1 cc portions were transferred to fresh litmus milk and incubated anaerobically with no growth apparent at the end of 10 days' incubation.) The ratio of viable aerobes to anaerobic spores in the same fecal emulsion before the diet was begun was approximately 5:1. The predominant organism in both the acetate agar and beef-liver agar was *B. coli*.

At the end of 4 days of the diet there was a pronounced change in the bacterial content of the fecal emulsions. At this time a streptococcus began to appear prominently in both the acetate agar and beef-liver agar plates, apparently replacing *B. coli*. Also, there was an enormous decrease in the relative numbers of anaerobic spores so that the ratio of viable aerobes to spores was 20,000:1. The smears consisted predominantly of gram-negative bacilli, although there was an increased proportion of slender gram-positive bacilli and diplococci.

This streptococcus was the predominant organism of the aerobes at the end of 7 days, and was found to ferment dextrose, lactose, maltose and mannite. Presumably it belonged in the streptococcus fecalis group as described by Oppenheim.<sup>20</sup> *B. welchii* was practically suppressed and remained so for the remainder of the experiment.

At the end of 10 days the flora was predominantly fermentative with the streptococcus dominating the field and the anaerobic spores eliminated, as shown by the fact that in a fecal emulsion containing 25 000,000 viable aerobes per cc there was less than 100 per cc of anaerobic spores. The stains showed gram-positive streptococci and slender bacilli occupying about 50% of the field, with only an occasional plump gram-positive bacillus present.

<sup>20</sup> Jour. Infect. Dis., 1920, 26, p. 117.

The diet was discontinued at this point, and a diet high in animal protein consumed. At the end of 3 days a fecal specimen collected as before was examined. *B. coli* was once more in predominance, and *B. welchii* was again present in large numbers in the anaerobic plates. The stains showed many plump gram-positive bacilli in a field which was composed predominantly of gram-negative bacilli.

#### HYDROGEN-SULPHIDE PRODUCTION

Hydrogen sulphide is formed in the intestines by the decomposition of cysteine and cystine. It is claimed by some (Mathews)<sup>21</sup> that this is readily reabsorbed and produces headaches and depression even when absorbed in small quantities. This author also suggests that it may be one of the factors in hemolyzing erythrocytes, thus causing anemia in those suffering from constipation. Mercaptans, such as ethyl and methyl mercaptan, are very ill smelling compounds that are also formed by the action of certain intestinal bacteria. Acute pathologic conditions due to the action of these substances appear to be quite rare, and with the exception of a case of intoxication with hydrogen sulphide of intestinal origin described by Senator,<sup>22</sup> no definite cases have been reported. Van der Bergh, however, has shown the presence of sulphemoglobin in the blood of persons with intestinal obstruction.

Torrey in his investigations found that members of the proteus group are very active producers of hydrogen sulphide, and that they were more abundant in the ileum than in any other part of the intestinal tract in the case of a dog on a lactose-meat-rice diet. He also found that saccharose, maltose and dextrose exercised some anti-putrefactive action, as evidenced by the decrease in the number of hydrogen-sulphide-producing organisms and *B. welchii*. These sugars were not as effective as lactose and dextrin, however. With a high animal protein diet (beef heart) the hydrogen sulphide producers, especially *B. proteus*, became numerous, and with a fish diet they seemed to be brought to development to a greater degree than with any other food element.

By the use of the lead acetate agar described, a marked difference in the relative abundance of these hydrogen-sulphide formers with different types of diet may be shown. Table 1 illustrates the difference in numbers with two diets, expressed in percentage of hydrogen-sulphide producing-organisms to total viable aerobes in the same fecal emulsion. One lot of 5 rats was fed a meat and carrot diet, while the

<sup>21</sup> Physiological Chemistry, Text-Book, 1915, p. 443.

<sup>22</sup> Wells, Chemical Pathology, 1918, p. 581.

other lot received the same diet with the addition of lactose in the proportion of meat 7 parts to lactose 3 parts. The platings were made after the animals had been on the diets for at least 2 weeks.

It is evident from table 1 that there is a material decrease in the number of hydrogen-sulphide producing organisms in rats on a meat and lactose diet.

The most typical brown colonies developing on the acetate-agar plates are members of the proteus group. Certain strains of *B. coli* also form distinct brown colonies at the end of 36 hours. Mr. R. W. Cooper and Miss Mirium Jackson made a somewhat detailed study of this point with the following results: Forty-four strains picked from

TABLE 1  
NUMBER OF HYDROGEN-SULPHIDE FORMERS PRODUCED WITH TWO DIETS

Meat Diet			Meat and Lactose Diet		
Rat	Total Bacteria per C c	H <sub>2</sub> S Formers per C c	Rat	Total Bacteria per C c	H <sub>2</sub> S Formers per C c
49.	5,240,000	80,000	34.	6,400,000	10,000
1.	360,000	30,000	6.	890,000	10,000
2.	80,000	20,000	7.	800,000	1,000
3.	310,000	10,000	8.	11,600	100
4.	3,700,000	70,000	9.	360,000	20,000
5.	610,000	140,000	10.	1,000,000	100
			6.	50,000	8,000
			7.	25,000	1,200
			8.	35,000	100
			9.	25,000	100
			10.	32,000	200
Total Percentage	10,300,000	350,000	....	9,628,600	50,800
	3.4		....	0.5	

acetate-agar plates from different rats on various diets were *B. proteus vulgaris*, giving the typical spreading growth on agar slants, liquifying gelatin, forming indol, and fermenting maltose, saccharose and galactose, with gas, but negative to lactose, mannite and inulin. One hundred brown colonies in plates from rats fed meat or salmon were picked and 50 were identified as *B. proteus*. On the other hand, the predominant brown colonies in plates from rats fed lactose and bread or egg yolk were members of the colon group. Ninety-four strains of the latter group were studied and found to be about evenly divided between saccharose-fermenting and saccharose nonfermenting types. In no case, however, was the formation of hydrogen sulphide as active as with the members of the proteus group.

*Anaerobes*.—No extensive analysis of the spore-forming anaerobes found in the intestinal tract of the white rat or man has been made in

the present study, but certain suggestive facts have been determined. For example, hydrogen-sulphide-producing anaerobes are found in large numbers in the feces of white rats when on a high animal protein diet and are greatly reduced in numbers or practically completely eliminated when lactose, certain vegetable proteins or starches are fed. These bacteria appear to be *B. welchii* in the majority of cases, as evidenced by the following experiments: Well separated brown colonies from the anaerobic plates have been transferred to litmus milk and incubated anaerobically. Eighty-nine such colonies from rats fed either meat, beans, egg yolk, milk or bread and lactose when picked to litmus milk gave typical stormy fermentation with butyric acid odor in 24 hours. About 75 of these were transferred to dextrose agar and shown to be anaerobes. Forty of those in dextrose broth were nonmotile by the usual motility test, of 42 tested. Twelve dextrose broth cultures (48 hours) were heated to 80 C. for 20 minutes and 1 c.c. portions added to litmus milk. Ten gave no growth in 10 days' incubation, while the cultures which showed motile bacilli gave a soft coagulum with a clear whey and no gas with a gradual peptonization of the curd. Nineteen milk cultures showing stormy fermentation (3 days' old) were heated in the same manner and 1 c.c. portions transferred to litmus milk. Seventeen gave no growth at the end of 10 days, while 2 coagulated the milk with a soft curd and peptonization.

From these tests it was concluded that in the white rats tested *B. welchii* was the most prominent of the spore-forming anaerobes, accompanied in smaller numbers by *B. sporogenes*.

The effects of the following food stuffs when fed alone for several days to white rats have been determined with reference to the development of hydrogen-sulphide producing organisms; ground beef, whole eggs, egg white, egg yolk, cheese, potato, lima beans, cow peas, English peas, salmon, bread, milk and lactose, and adiabatic flour (Hepco). These foods were fed in rotation to a series of rats, and the relative numbers of hydrogen-sulphide-producing organisms determined for each diet. The averaged results are:

Salmon: Four rats were fed salmon for from 1 to 4 weeks. The average of 12 platings during this period showed that 7.3% of the organisms growing on the acetate agar aerobically were producers of hydrogen sulphide. Approximately 200,000,000 colonies were counted in getting this percentage. These results agree with those of Torrey in that these hydrogen-sulphide-producing organisms were almost entirely *B. proteus vulgaris*. This fish diet also led to an enormous increase in the numbers of spore-forming anaerobes, especially *B. welchii*.

**Ground Beef:** Seven rats were fed ground beef for from 2 to 19 days. The average of 16 platings during this period gave a percentage of 7.5 for hydrogen-sulphide-producing organisms out of about 50,000,000 colonies counted. Hydrogen-sulphide-producing anaerobes were abundant in this diet, and of the spore formers *B. welchii* was predominant, although no more so than in the case of the fish diet.

**Diabetic Flour (Hepco):** This flour, according to the manufacturer, has as its base the Soya bean and contains approximately 43% protein, less than 23% carbohydrate with only a trace of starch, 21% fat and about 4.5% each of water, ash and fiber. This flour was fed uncooked to 7 rats for a period of from 2 to 7 days. The average of 8 platings from these animals showed that 0.05% of 50,000,000 colonies were hydrogen-sulphide producers. *B. acidophilus* was in predominance in all the plates and spore-forming anaerobes were almost completely suppressed. Even when this flour followed a diet that had led to a greatly increased percentage of *B. welchii*, the Hepco flour led to the elimination of *B. welchii* spores or reduced their numbers to a negligible quantity.

**Cow Peas:** The foregoing results with a diet high in vegetable protein suggested that perhaps this was a property of vegetable proteins in general, and although the legumes have almost 3 times as much carbohydrate as protein, nevertheless it was considered advisable to test out their effects as units on the intestinal flora. Cow peas which had been autoclaved at 15 pounds pressure for 20 minutes were fed to 5 rats for from 2 to 8 days. The platings during this period gave a percentage of 0.03 of hydrogen-sulphide-producing organisms out of a total of more than a billion colonies considered. This diet brought *B. acidophilus* in marked predominance to as great a degree as did the diet of meat and lactose. The spore-forming anaerobes were also completely suppressed by this diet, as for example in certain fecal emulsions in which there were 150,000,000 viable aerobes per c.c. with less than 100 spores of *B. welchii* per c.c.

**Lima Beans:** These were also autoclaved in an equal volume of water at 15 pounds pressure for 20 minutes and fed to 3 rats for from 6 to 8 days. The average of 6 platings during this period showed that 0.03% of the colonies developing were producers of hydrogen sulphide. Approximately 350,000,000 colonies were considered in getting this percentage. Here too, *B. welchii* and other spore-forming-anaerobes were practically eliminated.

**English Peas (Canned):** These were fed to 4 rats for 6 days and platings made at the end of that period. The average of this series gave a percentage of 0.2 of hydrogen-sulphide producers. This percentage was somewhat higher than with the other legumes largely due to the fact that rat 18 had a flora containing an unusually high proportion of *B. proteus*. Even in this rat, however, *B. welchii* was completely suppressed as it was in the case of the other 3 animals on this diet.

**Potatoes (White):** These were autoclaved at 15 pounds pressure for 20 minutes and fed to 5 rats for from 2 to 4 days. The average of 8 platings gave a percentage of 0.4 of hydrogen-sulphide producing organisms. The percentage at the end of 4 days of the diet was less than this, indicating that the high starch diet tended to eliminate hydrogen-sulphide producers. *B. welchii* and other spore-forming anaerobes were almost completely suppressed at the end of 4 days.

**Egg Albumin:** Six rats were fed coagulated egg white exclusively for from 4 to 10 days and gave an average of 3% of hydrogen-sulphide producers out of 200,000,000 colonies considered. Anaerobic spores were greatly increased

in numbers. Three of the rats on this diet died within 5 days, one in 6 days and another in 10 days. The rats were in separate cages, and as no deaths occurred with the other diets during a period of 2 months, it appears probable that there must have been some toxic effect from the unbalanced diet of egg white.

**Egg Yolk:** One rat only was tested. This animal was fed boiled egg yolk for one month. During this period 5 platings gave a percentage of 3.2 of hydrogen sulphide producers in 30,000,000 colonies examined. Anaerobic spores were increased in numbers also.

**Whole Eggs:** Six rats were fed scrambled eggs for from 4 to 12 days. Seven platings during this period gave 2% of hydrogen-sulphide formers. Anaerobic spores were also present in enormous numbers with this diet.

**American Cheese:** Six rats were fed cheese for from 2 to 3 days. Anaerobic spores were practically eliminated with this diet. The aerobic producers of hydrogen sulphide were present in 0.9% of the colonies counted. This is somewhat similar to the findings of Torrey that both casein and butter fat had little tendency to encourage the growth of putrefactive types.

TABLE 2  
C-A RATIO OF VARIOUS REGIONS

Rat	Diet	Stomach C-A Ratio	Duodenum C-A Ratio	Jejunum C-A Ratio	Ileum C-A Ratio	Cecum C-A Ratio	Colon C-A Ratio
A	Lactose, bread and milk	0*	1-99	1-99	1-99	1-99	15-85
B	Meat	0*	0*	0*	99-1	99-1	99-1

\* Less than 100 organisms per c c of emulsion.

**Bread, Milk and Lactose:** Seven rats were fed this diet for from 2 to 10 days. Equal parts of dried bread and lactose were soaked in milk and fed. The platings during this period gave a percentage of hydrogen sulphide producers of 0.06 out of 500,000,000 colonies considered. Anaerobic spores were almost completely eliminated by this diet.

#### INTESTINAL SURVEYS

Two rats, one on a diet of ground beef for 2 months and the other on a bread, milk and lactose diet for 2 weeks, were killed and under aseptic conditions dilutions of the contents of the stomach, duodenum, jejunum, ileum, cecum and colon plated to get an idea as to the relative distribution of *B. coli* and *B. acidophilus*. The emulsions from the different levels were adjusted as nearly as possible to the same turbidity to make the results somewhat comparable. The C-A ratio of the various regions is shown in table 2.

In the case of the animal on the high animal protein diet, we find both *B. coli* and *B. acidophilus* present in extremely small numbers in the stomach, duodenum and jejunum. *B. coli* appeared in large numbers in the ileum and was the predominant organism from there on. Torrey has noted that putrefaction, as judged by types and biologic activities of the bacteria, was more marked in the ileum than in the large intestine, and he suggested that this might be the case in persons suffering from toxemias of intestinal origin, to whom colonic irrigations afforded no relief.

The findings were reversed with the rat on a high lactose diet. Here we find large numbers of *B. acidophilus* appearing in the duodenum (80 000 per c c

as against less than 100 of *B. coli* from the same emulsion) and being in enormous predominance throughout the remainder of the intestinal tract. The emulsions from this animal were also plated into acetate agar. In the case of 1 c.c. amounts of the original emulsions from the stomach, duodenum and jejunum, not a brown colony appeared on the plates. A few colonies were present in the ileum, cecum and colon, but their number was insignificant when compared with the total number of *B. acidophilus* per c.c. in the same emulsions. In both cases, however, the first evidences of activity of proteolytic bacteria were found in the ileum.

Rat C. was fed lactose, bread and milk for 8 days. At the end of 5 days the feces were soft, a light yellow, practically odorless and showed no trace of indol. Stains showed a field composed almost entirely of slender gram-positive bacilli and a few short chains of streptococci. On the eighth day the animal was killed, and the various regions plated to determine the distribution of hydrogen sulphide producing organisms. Here *B. acidophilus* was found in large numbers from the duodenum on, while *B. coli* was practically absent. Hydrogen-sulphide formers were also in insignificant numbers throughout the intestinal tract.

#### DISCUSSION

A contrast in the tendency of animal and vegetable proteins to encourage putrefaction in the intestinal tract is clearly shown in these experiments. Torrey pointed out this peculiarity when he found that "vegetable proteins do not offer the slightest encouragement to the growth of the intestinal putrefactive types of bacteria." My experiments agree with those of Torrey in that vegetable proteins not only reduced the relative proportion of proteolytic types both aerobic and anaerobic, but also encouraged the overgrowth of a nongas-producing aciduric flora. Animal proteins, on the other hand, such as meat, fish and eggs, led to an enormous overgrowth of gas-forming proteolytic types.

The question of the absorption and excretion of materials from the intestinal tract has been recently studied by Underhill and Simpson.<sup>23</sup> These workers have found that the diets which give rise to the excretion of phenol and indican in large amounts are the ones that lead to the overgrowth of putrefactive bacteria in the intestinal tract. Meat led to a marked increase in the excretion of phenols and indican, whereas casein caused much less phenol and indican to appear in the urine. Lactose in the diet caused the excretion of phenol and indican to be lower than when large amounts of protein were fed. They found vegetable proteins to be on the same level as casein in regard to the excretion of phenol and indican. This work is a further indication that there is a definite correlation between the formation and the absorption of the by-products of bacterial activity in the intestinal

<sup>23</sup> Jour. Biol. Chem., 1920, 44, p. 69.



tract. It may be more than a coincidence that this increased absorption accompanies a gas-producing type of flora. It is a well-known fact that absorption in the intestine is increased by raising the intra-intestinal pressure, independently of the increase in mucosa surface. In an intestinal tract distended with gas both factors may be concerned and lead to an increased absorption that may be concerned in the production of mild or acute grades of toxemia. As pointed out, the detoxicating capacity of the liver is limited in acute intestinal obstruction, and there is evidence that it may be limited in some of the subacute and chronic toxemias that are apparently of intestinal origin.

#### SUMMARY

In this paper certain methods for ascertaining the relative proportions of groups of bacteria of the intestinal tract are described, particularly in studying the hydrogen-sulphide-producing organisms and the spore-producing anaerobes. By the use of these methods essentially the same results as those of Kendall, Rettger et al and Torrey have been obtained, namely:

Grain foods, lactose and dextrin when fed to albino rats in proper proportions lead to a marked predominance of aciduric bacteria in the intestinal tract, whereas animal proteins encourage the gas-producing proteolytic types, both aerobic and anaerobic.

Vegetable proteins and certain starchy foods do not encourage the development of proteolytic types to the same extent as animal proteins, and, in fact, in many cases exert a distinct antiputrefactive effect, favoring the development of *B. acidophilus* and suppressing the development of hydrogen-sulphide-producing organisms and spore-forming anaerobes.

In 2 experiments with human adults extending over a period of 10 days a diet composed of bread, milk and lactose markedly encouraged the development of the aciduric organisms, and in one experiment of the same time period a diet high in vegetable protein led to a predominant aciduric flora with the elimination of anaerobic spores.

# VIRULENT TREPONEMA PALLIDUM RECOVERED FROM A STILLBORN INFANT AFTER TWENTY-SIX HOURS

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Stillborn, congenitally syphilitic infants are the chief sources of material for the demonstration of treponema pallidum in tissues, because it is generally known that their organs commonly contain great numbers of the organisms. That the treponema occasionally retains its virulence for some hours after the death of the host, is not generally recognized and is, we believe, a new observation. Recently, we recovered virulent, actively motile, treponema pallidum from a stillborn infant 26 hours after it had been delivered, and we are reporting it to call attention to the fact that the chance of accidental infection from careless handling of syphilitic tissues is not as remote as is generally supposed. In this instance, the serum from superficial skin blebs and from crushed lung tissues were examined by the dark-field method, and great numbers of actively motile treponema were found. Rabbits, which were then inoculated intratesticularly with the material, developed the typical lesions of experimental syphilis.

The case is reported rather fully, since the mother's history presented some interesting features; the necropsy findings were somewhat unusual; and the well preserved state of the fetus may help to explain the retained virulence of the treponemas which it harbored.

A woman, married, white, aged 19 years, seven months pregnant, applied for admission to the hospital June 23, 1920, complaining of "wasting," and was delivered a few hours later. Family history unimportant. She stated she had always been well until about a year before when she had begun to have severe headaches, chiefly on the right side. About three months after the appearance of the headaches, she developed a general rash which first appeared on the forearms. She denied having had any venereal diseases but said she had been given eight injections of "salvarsan," though she had not had any during the last three months. Menstruation began at the age of 13, and occurred regularly. She had one child living and had had one miscarriage at 4½ months. Her last menstrual period was given as Nov. 22, 1919 (211 days before delivery). Blood taken for the Wassermann test, on admission, gave complete fixation with cholesterinized antigen, Neymann's antigen, and acetone insoluble antigen, + + + + reaction.

Received for publication June 1, 1921.

*Placenta*.—The placenta presented none of the classical gross or microscopic lesions generally attributed to syphilis, due possibly to the eight arsphenamin treatments.

*Fetus*.—Premature stillborn infant, apparently about the seventh month. The dead fetus, together with the placenta, was placed in the refrigerator over night and sent to the laboratory for necropsy on the following day. Necropsy begun 26 hours after death.

*Anatomic Diagnosis*. — Congenital syphilis—pneumonia alba, multiple abscesses of thymus, papillary and vesicular syphilides, epiphyseal osteochondritis.

The body was well developed, fairly well nourished, and free from the changes commonly seen in still-born infants which have been retained in the uterus for some time after death. There were many fine papules and a few well developed blebs on the hands and feet. These were quite as numerous in the palms of the hands and on soles of feet as on the extensor surfaces. A few of the blebs were broken and slightly ulcerated, but there was no real maceration. There were no deformities, anomalies or rugae about the mouth.

Most of the organs were normal in appearance. The liver and suprarenals were distinctly enlarged. The thymus gland contained numerous small yellow cheesy areas. The lungs were mottled, white and red in color, opalescent in appearance, resilient to touch, and resembled pancreatic tissue on section. The ends of the long bones showed uneven lines of ossification.

Silver impregnation preparations of all of the tissues by the Levaditi and by the acetone silver methods showed great numbers of treponemas in the lung tissues, both in the interstitial tissues and in the exudate which filled the alveoli and bronchioles. A few treponemas were found in the papules, and many more in small pustules which were situated in the corium underneath some of the superficial blebs. Moderate numbers were also found in the heart muscle, spleen, liver and suprarenals. Microscopically, the small cheesy masses in the thymus were pustules, not gummas, and repeated silver stains failed to show the presence of treponema in them.

The cerebrospinal fluid gave a + + + + Wassermann test.

Dark-field examinations of the serum from the blebs and from crushed lung tissue showed great numbers of typical treponemas. A loop of cerebrospinal fluid contained an occasional treponema, the motility of which was questionable.

Two rabbits received intratesticular inoculations with serum from the blebs and lungs; both developed indurated nodules, and one a diffuse orchitis in addition. The nodules were late in appearing, the earliest being noted about seven weeks after inoculation. Treponemas were recovered from both animals and others inoculated. Repeated transfers have been made from animal to animal, and after nine months the strain still remains pathogenic for rabbits.

#### DISCUSSION

Aside from the mere fact that stillborn congenitally syphilitic infants may harbor the active virus of syphilis, the evidence obtained in this case raises an interesting point, namely, the inconsistency between the presence after death of active virus in tissues and the absence of any

data on infection attributable to such material. There is a belief prevalent among laboratory men that *treponema pallidum* loses its virulence almost as soon as the host is dead, and that syphilitic tissues may be handled with impunity. Apparently, there is much negative evidence to support this view. The organs of syphilitic fetuses simply teem with *treponema pallidum*, as demonstrated by the use of various silver methods. Probably thousands of necropsies have been made on them by pathologists who did not wear gloves, and yet the statement that "There is not an authentic case of laboratory infection of syphilis on record," is often quoted. In this case we have an infant in whom actively virulent *treponemata pallida* were proved to be present. Here the pathologist stood a good chance of becoming infected if he had not been protected. The use of gloves, a whole unbroken skin, and the natural immunity which some persons appear to possess, may serve to explain the escape of many, but when one stops to think of the great number of exposures laboratory workers have had with so little evidence of infection, something additional seems to be necessary for a complete explanation.

We have been making a series of experiments which may throw some light on the problem, and while these are not entirely completed we have already elucidated the following points: 1. *Treponemas* obtained from excised chancers retain their virulence for about 24 hours, as shown by rabbit inoculation. 2. Inoculations with the same materials made after 36 hours have as yet been uniformly negative. 3. *Treponemas* kept in excised tissues retain their motility for several weeks, if protected from drying. 4. Pieces taken from excised tissues positive for *treponema pallidum* and treated by one of the silver methods show that the staining qualities are retained for weeks, though no preservative of any kind is used. Sometimes the *treponemas* appear to be even more numerous after several weeks than they were in the tissues examined at the time the material was excised. We have not as yet been able to prove to our own satisfaction whether or not saprophytic growth actually occurs in these tissues.

Applying the foregoing observations, we find that *treponema pallidum* usually loses its power to infect, after about 27 hours' existence in dead tissues, but that it does not lose its staining characteristics for a long time. As a rule, syphilitic fetuses are badly

macerated, showing that they have been dead in utero for a considerable length of time, so that it is probable that the treponemas which they contain have lost their virulence, though they have retained their staining qualities.

However, the demonstration of infectious treponema pallidum in one fetus makes it advisable to be on the safe side by adopting thorough measures for protection in examining all syphilitic tissues.

## ADVANTAGES OF CULTURE MEDIUMS CONTAINING SMALL PERCENTAGES OF AGAR

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The peculiar characteristics of agar have made it an important factor in the development of the science of bacteriology. Too frequently it is considered merely an inert constituent of culture mediums useful because of the changes in its physical state at various temperatures, while the effects on bacterial metabolism of different percentages of it are ignored. The maximum concentration in common use is fixed more by convenience in filtration than by economy or influence on growth.

It is true that semisolid mediums have been suggested from time to time. Rosenthal<sup>1</sup> and Klie<sup>2</sup> experimented with concentrations of gelatin as low as 2.5%. The luxuriant growth obtained probably as a result of diffusion of the inhibiting metabolic products and the extension through the soft jelly of the colonies of motile bacteria caused gelatin to be preferred by many workers and resulted in its being given up with reluctance in favor of the more convenient agar. Hiss<sup>3</sup> and later Hesse<sup>4</sup> found that 0.5% agar permitted flagellated bacteria to travel through it and suggested this medium as a means for differentiating between the motile typhoid bacillus and the nonmotile colon bacillus. Jackson and Melia<sup>5</sup> working with the Hesse medium found that about 0.4% of dried agar was the equivalent of the 0.5% recommended by those who had not taken into consideration the moisture content of thread agar. North<sup>6</sup> found that semisolid mediums were more suitable for preserving stock cultures, doubtless for the reason that unlike solid mediums they did not hold the concentrated products of growth in direct contact with the bacteria. The gelatin-agar medium, attributed by North to Guarnari, contains but 0.3% of agar. Lignières<sup>7</sup> has more recently made the claim that a semisolid medium containing but 0.25% of agar is superior to broth or solid mediums for the cultivation of anaerobic bacteria. That there are advantages in still lower percentages than have heretofore been employed has been revealed in the work reported in the following. The extreme simplicity of such a procedure has caused me to feel that it could not be new to any one but myself, and I have sought diligently to find references to previous work along the same line, but so far unsuccessfully.

Received for publication June 6, 1921.

<sup>1</sup> Deutsch. Arch. f. klin. Med., 1895, 55, p. 513.

<sup>2</sup> Centralbl. f. Bakteriöl., I, O., 1896, 20, p. 49.

<sup>3</sup> Jour. Exper. Med., 1897, 2, p. 677.

<sup>4</sup> Centralbl. f. Bakteriöl., I, O., 1908, 46, p. 89; Ztschr. f. Hyg. u. Infektionskr., 1908, 58, p. 441.

<sup>5</sup> Jour. Infect. Dis., 1909, 6, p. 194.

<sup>6</sup> Jour. Med. Research, 1909, 20, p. 359.

<sup>7</sup> Compt. rend. Soc. de biol., 1919, 82, p. 1091.

Our interest in this subject was stimulated by seeing colonies of *Clostridium histolyticum* in deep agar which were considered by J. F. Donner, late lieutenant, Sanitary Corps, U. S. A., entirely atypical, in that they are unusually large and filamentous. In seeking an explanation it was decided to study some of the factors which might influence colony formation. The first of these taken up for investigation, because it seemed and has proved to be one of the chief causes of variation, was concentration of the agar.

In the study of the pathogenic anaerobic bacteria, the type of colony formed in the solid agar medium used is considered a valuable differential characteristic. Usually, however, no statement is made in published reports concerning the percentage of agar used in the medium, and we seldom find attention paid to the fact that the thread agar of commerce contains impurities, and, according to Whitaker<sup>8</sup> from 18 to 20% of moisture. Ayers, Mudge and Rupp<sup>9</sup> have recently called attention to the influence on bacterial growth of some of the impurities in commercial sugar which may be largely removed through washing, as noted by the Committee on Standard Methods of the Laboratory Section of the American Public Health Association.<sup>10</sup>

#### PREPARATION OF CULTURE MEDIUM

The culture medium selected for the study of colony formation was similar to the putrid meat medium of Veillon.<sup>11</sup> In order to have the various lots, containing the different percentages of agar, uniform in every other respect, double strength broth was made, and to this was added an equal volume of agar in distilled water. One kilo of ground lean beef stirred into 1 liter of water was incubated at 37 C. for 48 hours. This was then strained and heated in a water bath to boiling and strained again. Then were added peptone, 40 gm. (2% in the final medium) and  $\text{KNO}_3$ , 4 gm. (0.2% in the final medium). When the peptone had dissolved, the broth was titrated and the reaction adjusted to  $\text{P}_H$  7.5. It was then filtered and autoclaved at 15 lbs. of pressure for 30 minutes. Just before mixing with the agar, enough glucose was added to make 0.2% in the final medium.

To make the agar jelly, the thread agar was dried thoroughly, weighed, washed in running water over night and made up with distilled water to 6%. The agar was dissolved in the autoclave, reaction adjusted to  $\text{P}_H$  7.5 and cleared by straining through cotton and gauze. In making the mixtures the amount of hot, fluid, 6% agar necessary to obtain the various percentages was diluted with hot distilled water to a volume equal to that of the double strength broth—also hot. For example, to make 500 c.c., 1% nutrient agar and to make 83 c.c., 6% agar was mixed with 167 c.c. distilled water and 250 c.c. double

<sup>8</sup> Jour. Am. Pub. Health Assn., 1911, 1, p. 632.

<sup>9</sup> Jour. Bacteriol., 1920, 5, p. 589.

<sup>10</sup> Standard Methods for Examination of Water and Sewage, 1920, p. 93.

<sup>11</sup> Arch de med. et pharm. mil., 1918, 69, p. 15.

strength broth. The reaction was checked, the medium was tubed without further filtration and the tubes were sterilized in the autoclave at 15 lbs. of pressure for 20 minutes. Before inoculation the reaction was again checked.

#### TECHNIC OF INOCULATION

For inoculation, the tubes of medium were heated in a water bath for one-half hour and chilled rapidly to about 45 C. A 24-hour meat broth culture served as the seed. A sterile glass capillary sealed at the end was dipped into the broth culture and carried successively through 9 tubes of the agar without reinoculating. The glass inoculating rod was inserted into the warm fluid and moved about carefully to avoid whipping air into the medium. By this dilution method of inoculating, the first 3 tubes generally showed innumerable colonies, while succeeding tubes showed a rapidly diminishing number. Nearly always, one or two of the tubes contained only from 1 to 5 colonies.

#### PERCENTAGE OF AGAR AND COLONY MORPHOLOGY

The strain of *C. histolyticum* in question, from a single organism, isolated by Lieut. Donner with the Barbour apparatus, usually produces colonies like the illustration shown in Weinberg and Seguin, plate 5, figure 5.<sup>12</sup> The naked eye appearance of these colonies is much like a crumb of very dry bread. That is, they are irregularly star-shaped and have a fairly sharp contour.

In this series, the colonies in agar of from 1.25% to 3% developed the typical bread crumb morphology. In the 1% agar the colonies became much larger and spherical in shape, growing radially from a central nucleus. They finally became fairly dense and regular in contour, and had somewhat the puff-ball appearance certain of the molds assume when growing suspended in broth. These colonies are shown in Fig.1. The largest of the colonies in deep agar measured about 10 mm. in diameter. This was in the lower half of the tube, the one other colony in the culture being in the upper half with a distance of nearly 15 mm. between. Both colonies were identical in appearance and the upper was used to confirm the identity of the culture; this was done by microscopic examination, sugar reactions, colonies of subcultures in higher percentages of agar and finally by animal inoculation.

The colonies in the 0.75% agar were similar—possibly less dense. One of the 0.75% tubes contained nearly 100 colonies. These exhibited no tendency to coalesce; in fact, colonies approaching one another were flattened somewhat, with a narrow layer of clear agar between, as though the products of their growth inhibited further extension.

The 0.5% and the 0.1% agar showed a most luxuriant diffuse growth; the former was nearly solid while the latter seemed quite fluid.

<sup>12</sup> Le Gangrene Gazeuse, 1918, p. 169.



In none of the cultures was there any growth within 5 mm. of the surface; this superficial layer, probably invaded by oxygen, was not clouded to the slightest degree.

As noted in the foregoing, various amounts of agar, less than enough to make a firm, solid medium, have been recommended from time to time. The medium of Guarnari, containing 0.3% and that of Lignières<sup>7</sup> containing 0.25%, apparently reach the lowest proportions used and investigated heretofore. It is clear from our work, however, that these workers in stopping at 0.3% and 0.25% did not exhaust the advantages of dilution, nor did they reach the optimum point.



Fig. 1.—Colonies of *Clostridium histolyticum* in Veillon agar; incubated 48 hours; A, 2% agar; B, 1% agar.

The growth of *C. histolyticum* in the 0.1% agar was so luxuriant, it was decided to study the suitability of the lower percentages for the growth of both aerobes and anaerobes and to observe the relation of agar in various concentrations to the penetration of oxygen, using methylene blue as an indicator.

#### THE GROWTH OF ANAEROBIC BACTERIA IN 0.1% AGAR

*Primary Cultivation.*—A point of interest in this connection was the possible value of nutrient thin agar as an enriching medium or for the

primary culture directly from the infected wound or for other infectious material. If it might be found useful for this purpose, its simplicity and ease of preparation would be strong points in its favor.

Six samples of earth were collected from various places and planted into freshly heated, plain beef-infusion-broth-0.1%-agar, reaction PH 7.6, without glucose. In 18 hours the growth was heavy; microscopically, few spores were found; these were oval and somewhat rectangular. After 5 days every flask contained round spores and many bacilli morphologically like tetanus. Small quantities of the lower layers of the culture were removed and heated in sealed capillary tubes to 70 C. for 30 minutes. The heated material was planted into freshly heated 0.1% agar. At the end of 24 hours' incubation, the growth was luxuriant. Small amounts were passed through Mandler filters and 0.5 c c of each was injected subcutaneously into guinea-pigs. Two of the 6 animals died with symptoms of tetanus in about 48 hours.

*Stock Cultures.*—Some of the war wound anaerobes in the collection at the Army Medical School have been planted in 0.1% agar and all have grown well; these include *Clostridium tetani*, *C. welchii*, *C. histolyticum*, *C. septicum* (*Vibrio septique*), *C. edematicum*, *C. purificum*, *C. sporogenes*, and also types A and B of *C. botulinum*. The ease with which cultures of anaerobic bacteria in this medium may be successfully inoculated, makes it possible to study their physiologic characteristics as easily as those of aerobic bacteria. Noteworthy is the simplicity with which the carbohydrate relations of the various anaerobes may be observed.

*Toxin Production.*—Since the anaerobic bacilli grow well in this medium, apparently more luxuriantly than in broth, interesting possibilities are suggested with regard to toxin production. In preparation for work along this line methods for eliminating the agar, in case its thorough removal should be found necessary, have been investigated. If only small amounts are being prepared, the agar may be packed by centrifugation and the supernatant broth decanted. This will remove most of the agar but not all and if the toxin should be intended for intravenous injection, it might not be adequate unless centrifugation is repeated two or three times. For larger amounts such as may be used for the immunization of horses to produce antitoxins and for more thorough elimination of the agar, the culture may be precipitated by the addition of an excess of ammonium sulphate. The agar will be thrown out with the toxin. The precipitate obtained may then be

kneaded to eliminate as much salt as possible and dried over sulphuric acid in vacuo. On dissolving the desiccated mass the toxin will go into solution and the agar, before it has swelled to any appreciable extent, may be removed by filtration.

#### THE GROWTH OF AEROBIC BACTERIA IN 0.1% AGAR

In order to ascertain the range of suitability of the medium for the more common aerobic bacteria, flasks and tubes were prepared and

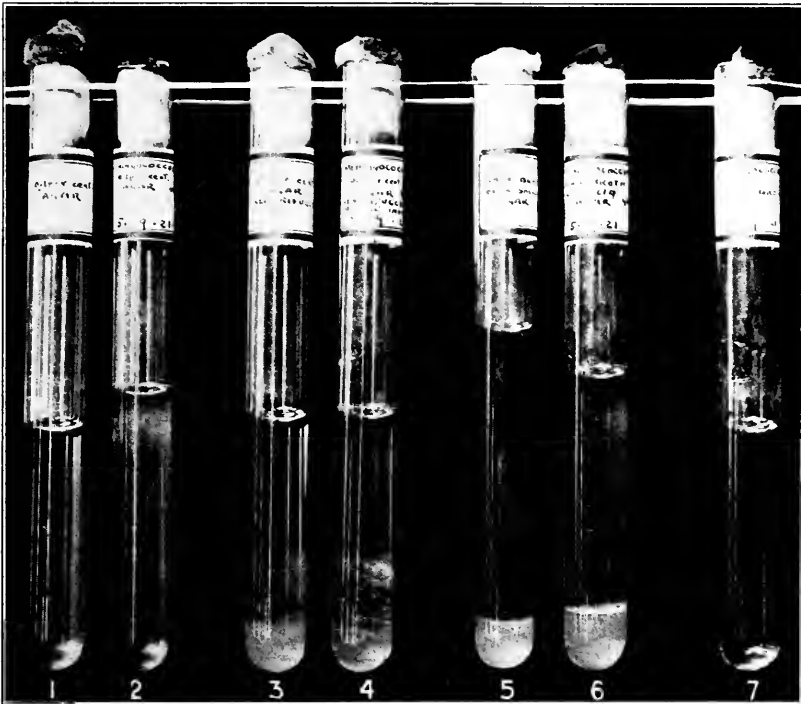


Fig. 2.—(a) Control broth. Tube 7 shows no growth, although planted at the same time. (b) Undisturbed 0.1% agar. Tube 1, uninoculated, shows clear broth above the slightly cloudy agar. Tube 2, inoculated, shows heavy granular cloud at upper level of agar with clouding of broth above. (c) 0.1% agar packed by centrifugation. Tube 3, uninoculated, shows deeper layer of clear broth with agar in the bottom of the tube and masses of agar floating above. Tube 4, inoculated, shows particles of agar accentuated by the growth surrounding them and slight clouding of the broth above. (d) Not illustrated. The appearance of the tubes was similar to "C". (e) Solid agar with control broth above. Tube 5, uninoculated; tube 6 shows growth on the surface of the agar and clouding of the broth above.

planted with various stock cultures. Naturally, any of the organisms which grow well in ordinary broth grew well in the 0.1% agar. In many instances growth in the latter was more luxuriant than in broth

or on solid agar. Several of the common contaminants from air and water, members of the colon-typhoid-dysentery group and the gram-positive cocci all grew well; then some of the more delicate bacteria were studied.

The meningococcus and the gonococcus grow most luxuriantly and in a manner which promises to add something of value to the cultivation and study of these gram-negative cocci. The luxuriance of growth suggested contamination so strongly that it was necessary to spend much time in the repeated examination of the cultures to rule out this factor. It should be added that the growth of these cocci is possibly no more luxuriant in the weak nutrient agar than in 1% starch in broth, but in the latter medium both organisms grow without differential characteristics and apparently never form pellicles. The starch jelly, however, settles out just as the agar does, and the growth seems to occur chiefly on its surface. Vedder<sup>13</sup> called attention to starch as favoring the growth of the gonococcus. The similarity of relations between the growth of these gram-negative cocci on starch and on agar is striking in view of the chemical and physical similarity of the two carbohydrates.

The meningococcus settles on and develops about the particles of agar, but it also grows as a diffuse cloud in the fluid above the agar; after 24 or 48 hours' incubation, pellicle formation commences.<sup>14</sup> The pellicle increases in thickness until it falls of its own weight or when the tube is disturbed. The growth is exclusively aerobic. Some recently isolated strains grow as well as do the old laboratory strains.

Some studies have been made with a view to learning more about the function of the agar and its relation to the growth. The same lot of broth and the same lot of agar jelly were used throughout. The 0.1% agar and the control broth, treated in several ways and then inoculated, gave the following results noted and illustrated in Fig. 2. The purity of these cultures was checked by plating and by microscopic examination after staining by Gram.

(a) Control broth; no growth except of some of the more hardy strains

(b) Undisturbed 0.1% agar; heavy growth at the surface of the agar and in the supernatant broth

<sup>13</sup> Jour. Infect. Dis., 1915, 16, p. 385.

<sup>14</sup> For the collection of recently isolated strains, I am indebted to Miss Alice C. Evans, of the Hygienic Laboratory, U. S. P. H. S. One of these strains, "HL-412" grows, not in the supernatant broth, but, like the gonococcus, exclusively about the particles of agar. It ferments glucose and maltose, but not sucrose, lactose or mannit.

(c) 0.1% agar packed by centrifugation, leaving a deeper layer of broth above; gradual accentuation in outline of the particles of agar; finally good-sized grayish white granules resulting from growth on these particles; gradual clouding of the broth above.

(d) The supernatant broth from tubes centrifuged as in (c); the first attempts to remove the supernatant broth without carrying over some of the agar, were not successful. In the tubes containing a few small particles at the bottom of the tube, the results were identical with those noted under (c). A second attempt was apparently more successful, but at the bottom of even these tubes there were a few masses, almost transparent, about which there seemed to be some growth, and the broth above was slightly cloudy. In these tubes there was definite growth, but it is not possible to rule out absolutely the presence of agar.

(e) The agar remaining in the tubes after removal of the supernatant broth (d) was heated and chilled, making a homogenous solid mass in the bottom of the tube; above this was carefully placed 10 cc of the control broth; gradual accentuation of the surface of the agar with definite colony formation there and slight clouding of the supernatant broth.

The luxuriance of growth in the tube containing undisturbed 0.1% agar, the relation of growth to the agar in the tubes which had been centrifuged and the very sparse growth in the removed supernatant broth can leave no doubt on the point at issue. The agar itself forms a kind of trellis for the coccus and thereby furnishes conditions favorable to its development. Whether or not the meningococcus actually utilizes the agar in its metabolism, we do not know.

Tubes of 0.05% agar, to which had been added several of the carbohydrates and the Andrade indicator, were inoculated with various strains of the meningococcus. The change in color was somewhat slower in developing in glucose than in maltose, requiring 72 hours for some strains. Acid formation was apparent with most of the strains tested within 48 hours. Tubes containing sucrose, lactose, galactose and mannit showed no change in color.

The viability of the meningococcus in mediums containing sugars is interesting. Apparently, in contradistinction to certain other organisms, some of the sugars, especially those which are fermented with the formation of acid, act in some way to prolong the life of the organisms. Possibly they exert a protein-sparing action. After 14

days' incubation of the 14 strains tested subcultures to blood agar from sucrose showed none living; from lactose, 1 living; from mannit, 1 living, from galactose, 9 living; from maltose, 11 living; from glucose, 13 living. The average reaction of the cultures after 16 days' incubation was: sucrose, lactose, mannit and galactose, PH 8.2; glucose, PH 6.8; maltose, PH 5.8. The reaction before inoculation was about PH 7.5.

In the supernatant broth of the meningococcus cultures, there are substances poisonous to white mice.

The gonococcus does not regularly cloud the supernatant broth, but grows either on or within the upper layers of the agar. Particles of culture pushed down toward the bottom of the tube apparently do not develop; in other words, the gonococcus, except in highly nutrient mediums, prefers to grow neither aerobically nor anaerobically. The growth is from the first granular; it seems as though the gonococci, unlike the meningococci, grow exclusively in contact with the particles of agar. As in the case of the meningococcus, pellicle formation is seen, however, after 24 or 48 hours.

The virulence or toxicity of these extremely rich cultures deserves investigation.

The two explanations for pellicle formation which immediately present themselves are: first, that the organisms have exhausted the oxygen supply and find at or just below the surface of the broth the exact conditions they need; the other is that the location of growth is related in some way to surface tension and, as suggested by Larsen,<sup>15</sup> with increasing surface tension, resulting from growth, pellicle formation is favored.

The Army Medical School gonococcus strain 3 at the end of 20 days in a 250 c c flask containing about 200 c c 0.1% agar showed heavy granular growth and pellicle formation. Subcultures demonstrated the purity of the growth. The fluid between the pellicle and the agar below was clear. Tested by Captain Williams it gave a positive complement-fixation test with antigonococcus rabbit serum and (commercial therapeutic) antimeningococcus serum, and with the rabbit serum it gave a positive precipitin test, using the technic of Robinson and Meader.<sup>16</sup>

<sup>15</sup> Abs. Bact., 1921, 5, p. 2.

<sup>16</sup> Jour. Urol., 1920, 4, p. 551.

The tubercle bacillus did not grow on 0.1% agar without glycerol but it has grown luxuriantly on the medium containing 4% glycerol. The pellicle did not start to sink until it was approximately three times as thick as the maximum growth commonly seen on glycerol broth. When it did begin to fall, at the end of about 6 weeks, it settled only to the surface of the agar. The center was sufficiently supported to keep it above the fluid and at 2 months it seems the bacilli are still multiplying. It is impossible to avoid speculation with regard to the possibilities of developing antigens in such cultures.

The acne bacillus has been grown recently by Captain Davis in 0.1% agar. He tells me the growth is best if he adds glucose and glycerol to the medium, and that the development of the culture is far more luxuriant than he has been able to obtain on any of the other mediums suggested for this organism. The appearance of the growth is interesting. Those bacilli which on inoculation settled to the surface of the agar, grew there, and becoming heavier gradually sank straight downward through the agar. In settling they left paths of growth resulting in a series of perpendicular lines. The part of the growth above the agar is slightly pigmented so that the entire culture looks like a sheaf of much elongated comedones!

#### TESTING VACCINES AND SERUMS FOR STERILITY

It is clear from the foregoing that in 0.1% agar we have a medium peculiarly suited to the detection of bacterial contamination, whether it be aerobic or anaerobic. No special apparatus, technic or culture medium is required, and the growth obtained is likely to be more luxuriant and therefore less easily overlooked, than with the use of current methods. The method is specially applicable to the examination of those substances which, like the so-called biologic products, contain a percentage of antiseptic.

In the preparation of the medium for this purpose, it is sufficient to add the proper amount of a previously prepared and assayed agar jelly to stock broth and sterilize. With careful technic the sterile agar may be added to hot sterile broth (as we frequently add some of the less staple sugars) and used without further heating. If the medium is prepared ahead, it should, of course, be placed in a water bath or Arnold just before use to drive off suspended oxygen, and then chilled rapidly. It is our custom to control the efficiency of the heating by

placing a few drops of 1% aqueous methylene blue in an extra flask or tube; heating is continued until the methylene blue is decolorized, except for the surface of the medium.

The amount of vaccine or serum to be planted in a tube or flask of 0.1% agar depends on its content in phenol or trikresol. The preservative must be diluted to a concentration of not more than 0.01%. The Army Medical School vaccines contain 0.25% of trikresol; the maximum amount that may be added to the culture medium is therefore 1 in 25.

In applying this method in the course of our routine work it has been used up to the present as an addition to the regular tests and not as a substitute for them. Every indication so far points to its being entirely dependable.

#### THE PHYSICAL PROPERTIES OF LOW PERCENTAGES OF AGAR IN NUTRIENT BROTH

In seeking an explanation for these results, chemical differences between solid and semifluid agar mediums do not seem to come under consideration. This is emphasized by the luxuriant growths obtained in the water of condensation of an agar slant where the constituents are qualitatively identical. The factors of importance must therefore concern differences in the physical condition of mediums containing high and low percentages of agar. Consequently, some attention has been given to these differences with the results recorded in the following.

We have made no attempt to prepare ash-free or electrolyte-free agar or to remove the nitrogen, of which crude agar contains 6.3%<sup>17</sup> according to Bordet and Zunz.<sup>18</sup> It is to be expected that different lots of crude threads even after washing will vary in jellying power and consequently in the size of the gel mass formed at the same percentage concentration. This is true of a lot of purified and powered agar, of foreign manufacture, that has recently come into our possession. Its jellying power is about twice that of the lot of thread agar used in this work. In the earlier observations with 0.1% agar in broth we had noted that agar is not miscible with broth in all proportions but below a certain point separates as a gel leaving the clear broth above. Attention seems not to have been called to this phenomenon in culture

<sup>17</sup> This figure is surisingly high. If it is correct, it seems likely that some of the N must be present in some other form than as protein N.

<sup>18</sup> Ztschr. f. Immunitäts., I, O., 1915, 23, p. 49.



mediums heretofore. Bordet and Zuns,<sup>19</sup> in their work with purified agar—pararabin—prepared by the method given by Grafe,<sup>20</sup> noted a separation but apparently this was more in the nature of a flocculation. They say that at 0.5% the upper part of the fluid is clear, while abundant whitish floccules are deposited and that ordinary agar is more coherent and more transparent. C. R. Smith<sup>21</sup> makes practically the same statement with regard to gelatin.

Ash-free gelatin swells in water to about 7 or 8 volumes. If such a gelatin is melted and cooled a clear, stable jelly is produced. If, however, a weaker jelly is prepared, syneresis takes place, with the production of a cloudy jelly. One-half per cent. jelly will flocculate into jelly particles (probably 7 times swollen) and can be filtered off completely from the extruded water which shows no traces of gelatin.

In the case of low percentages of agar in broth the process is distinctly that of the formation of a gel leaving the clear broth above. The relative viscosity of plain and glucose broth and of the extruded broth from 0.05% and 0.06% plain and glucose agar was determined by permitting equal volumes to fall through a capillary tube, the time being measured in seconds. The supernatant broth has the same viscosity as that of the control broth, of the same lot, to which no agar has been added. This is an indication that, like gelatin, agar is not soluble in water at temperatures below the point at which it gels. Sometimes floccules are seen just above the jelly mass, and the gel may be broken up into flakes by shaking the tube. The gel is at first only little less clear than the broth; later, after standing around for a few days and especially if shaken to break up the gel, it may become slightly cloudy. With clear mediums, the separation may be almost invisible. If the jelly is broken up by whipping it with a capillary glass rod, the flakes settle more compactly and occupy less volume than did the undisturbed gel. Once broken up in this way there is, of course, no change in the physical state of the agar until it is heated to near the boiling point.

In the solid mediums, containing at least 1% of agar, the colloidal particles are relatively closely packed together; in fact, this is true down to a concentration of about 0.2%. The squeezing out of fluid is noted in all agar mediums; this fluid constitutes most of what we call water of condensation. It seems from our work that, at 0.2% and less, the colloidal particles having reached the limit of their dissociation do not naturally remain further apart but that equal amounts of agar

<sup>19</sup> Loc. cit., p. 42.

<sup>20</sup> Abderhalden, E.: *Biochemisches Handlexikon*, 1911, 2, pp. 27 and 73.

<sup>21</sup> *Jour. Am. Chem. Soc.*, 1921, 43 (in press).

form gel masses of equal volume regardless of any increase in the volume of broth. On standing, the gel continues to contract and may not reach its equilibrium for several days. It is rather easily packed by centrifugation, and the weight of a luxuriant growth may force it downward. It is understood that this applies only to the conditions of temperature and composition and reaction of the broth with which we have been working. We believe it will apply proportionally to all ordinary lots of agar, but the relative amounts necessary to obtain the desired synaeresis may need to be ascertained for each lot. At 0.06% of our present lot the gel finally occupies about one-half the volume of the medium. This holds for test tubes of about three-fourths inch diameter. The separation in flasks is apparently greater since the greater bulk in shrinking leaves a relatively deeper layer of broth above. At present, we prefer 0.07% of agar for test tubes and 0.1% for 250 c c Erlenmeyer flasks.

The impression that the gel is in a state of equilibrium and therefore constant in density was formed by studying the appearance of tubes in series containing graduated amounts of agar and methylene blue. The use of this dye as an indicator of anaerobiosis has received much attention recently, notably by Hall<sup>22</sup> and Gates and Olitzky.<sup>23</sup> The following percentages of agar were made up (with 1% peptone, beef infusion broth, 0.5% sodium chlorid) according to the method given in the foregoing: 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.15, 0.20, 0.25 and broth controls; in repeating these observations we started with 0.01 and went up to 2.0%; the results were the same, the tubes outside this range added nothing of value or interest. These were filled into three-fourths inch test tubes, 15 c c to each, and before sterilization, 0.1 c c 1% aqueous solution of methylene blue was added to 3 tubes of each percentage. One series received 1% of glucose, the other received none. After sterilization in the autoclave for 15 minutes the reaction of the glucose series was a little higher than P<sub>H</sub> 7.4; that of the plain series a little less than P<sub>H</sub> 7.6.

The tubes were chilled on removal from the autoclave and placed in the incubator. The depth of the blue color was measured at intervals and the tubes have been retained up to the present. The glucose exhibited clearly its reducing effect; the broth without agar required 48 hours to lose its greenish color and take on the blue of the dye. The glucose-free broth became quite blue in 18 hours.

<sup>22</sup> Jour. Bacteriol., 1921, 6, p. 1.

<sup>23</sup> Jour. Exp. Med., 1921, 33, p. 51.

The appearance of the tubes containing agar was increasingly interesting from day to day. Except for the fact that the glucose tubes always showed more reduction and less penetration, there was no noteworthy difference. It was formerly my belief that the agar in uniform suspension throughout the broth acted merely as a hindrance and that the rate of diffusion of the oxygen depended on the relative solidity or fluidity of the semisolid medium as determined by its agar content. This impression is permitted by Gates and Olitzky,<sup>23</sup> who, in commenting on their results, make the following statement: "Even so small an amount as 0.02% of agar may inhibit the diffusion of oxygen to the depths of the culture tube, or at least so retard it that dextrose broth is able to maintain anaerobic conditions below a certain level. This level occurred at 1 cm. from the surface when 0.5% of agar was employed." It seems worth while to quote their table since the results obtained so nearly coincide with ours:

TABLE 1  
FORMATION OF OXYGEN INTO SEMISOLID MEDIUM

Tubes	Amount of Agar, %	Penetration of Oxygen from Surface of Medium		
		After 5 Hours, Cm.	After 24 Hours, Cm.	After 48 Hours, Cm.
1.....	0	1.0	4.0	Complete
2.....	0.02	1.0	1.5	3.3
3.....	0.04	0.9	1.2	2.2
4.....	0.06	0.9	1.1	1.3
5.....	0.1	0.8	1.0	1.1
6.....	0.2	0.8	1.0	1.1
7.....	0.5	0.7	0.9	1.0

While inspecting the first set of our tubes, 42 hours after removal from the autoclave, an important point was noted which these authors had failed to record. This refers to the synaeresis of the agar and its relation to the diffusion of oxygen into the medium. The penetration of oxygen is not only retarded, it is stopped or controlled by the agar in glucose broth. When the culture medium is still hot from the autoclave or water bath, the particles of agar are diffused through it as in a solution, but as it cools gel formation occurs in very low concentration, agar being insoluble at room and incubator temperatures; the gel mass contracts leaving the clear extruded broth above. The percentage of agar in the broth determines the relative volume of the gel mass; at 0.2% and above the entire volume of the medium is occupied. Below this point, the colloidal particles of agar tend to draw together to an equilibrium, and regardless of the relative amounts of agar and

broth the density of the gel is finally the same. The gel in this state permits the penetration of oxygen into the medium. As the gel contracts, the clear broth above becomes colored and the limit of contraction is reached only after several days. These points are illustrated in table 2.

The readings after 40 days in the incubator show that the agar, for practical purposes, permanently excludes oxygen. In the higher

TABLE 2  
PENETRATION OF OXYGEN INTO CULTURE MEDIUMS CONTAINING SMALL PERCENTAGES OF AGAR AND 0.1 C C OF A 1% AQUEOUS SOLUTION OF METHYLENE BLUE—  
AUTOCLAVED, CHILLED, INCUBATED

Per- centages of Agar	1% Glucose					No Glucose				
	18 Hours	42 Hours	72 Hours	5 Days	40 Days	18 Hours	42 Hours	72 Hours	5 Days	40 Days
Control broth	Green	Blue green	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
0.05	0.9	2.0*	3.3	4.2†	Blue†	1.3	3.2*	4.4	5.5†	Blue†
		0.7	0.7	1.5			1.8	2.0	2.5	
0.06	0.9	1.5	2.5	3.0	‡	1.3	2.2	3.3	3.7	Blue†
		0.7	0.7	0.8			1.5	1.5	1.6	
0.07	0.8	1.3	2.3	2.8	‡	1.3	2.0	2.8	3.6	‡
		0.8	0.7	1.0			1.5	1.3	1.8	
0.08	0.8	1.1	1.6	2.0	1.5	1.3	1.8	2.4	3.0	3.0
		0.8	0.7	1.0	‡		1.4	1.3	2.0	‡
0.09	0.8	1.0	1.3	1.6	1.5	1.3	1.7	2.1	2.6	2.8
		0.7	0.8	1.0	0.5		1.5	1.3	1.6	0.8
0.1	0.8	1.0	1.3	1.5	1.6	1.3	1.8	2.1	2.3	2.5
		0.6	0.8	0.9	0.5		1.7	1.3	1.6	1.2
0.15	0.8	1.0	1.2	1.4	1.4	1.3	1.7	1.9	2.1	2.7
		0.8	0.7	1.0	0.7		1.7	1.7	1.8	1.7
0.2	0.8	1.0	1.1	1.3	1.4‡	1.3	1.7	1.9	2.2	2.3‡
		1.0	1.1	1.3	1.4		1.7	1.9	2.2	2.3
0.25	1.2	1.6	1.9	2.2	2.5	1.3	1.6	1.8	2.1	2.4
		1.6	1.9	2.2	2.3		1.6	1.8	2.1	2.4

\* The upper figures represent the total depth of penetration of the oxygen, the lower, the depth of penetration into the agar, as shown by the return of color to the methylene blue. The measurements are expressed in centimeters and are taken from the bottom of the meniscus downward.

† Exact measurements are difficult on account of the separation of the agar from the sides of the tubes.

‡ Unsatisfactory for exact measurement chiefly on account of evaporation.

concentration the Liesegang rings, noted by Hall,<sup>22</sup> are present. These may easily be due to changes in temperature especially at times of removal of the tubes from the incubator. For comparison with the earlier readings the figures are of little value for there has been about 40% evaporation, the tubes have been brought out for examination repeatedly and the relations between agar and broth considerably disturbed. The agar, in the tubes containing 0.05%, is so small in amount it is difficult to tell how far the agar has been penetrated since it is almost surrounded by the colored broth. In the tubes containing

0.25%, the agar is penetrated farther and the color seems to be slowly advancing, in the 0.06 to the 0.2% tubes a point, practically stationary, was reached within five days. Agar itself in distilled water or in physiologic salt solution exhibits no reducing effect on methylene blue.

#### DISCUSSION

There are two outstanding points which make 0.1% agar of interest as a practical culture medium for the growth of bacteria. The first of these concerns the superior anaerobic qualities of the thin agar jelly, the second concerns not only the greater luxuriance of growth of many of the bacteria but also the successful cultivation of certain species which grow only with difficulty or not at all on the ordinary mediums.

With regard to the first point, our conception of the physical state of the agar seems to offer an explanation. When meat infusion broth containing agar is heated to near the boiling point, the agar goes into a state of solution and remains in this state until the temperature falls to slightly below 40 C. On cooling, the agar gels and the phenomenon of synaeresis is exhibited by the extrusion of a certain amount of the broth. The agar, insoluble in the broth, separates to form a gel in which the colloidal particles are in a state of equilibrium and apparently in the dispersing phase with the broth in the dispersed phase. This gel resists the penetration of oxygen since the undisturbed films of agar supported by the reducing action of the peptone and glucose offer a barrier to its advance. It is conceivable that in the case of solid mediums, the relations between the particles of agar and broth are modified by forces which act to destroy or fracture the continuity of the agar films. Thus, the broth, in a less confined state, is permitted to aid the penetration of oxygen but is likely to do so irregularly, depending on the degree of disturbance that has occurred in the process of cooling. The results obtained by Hall,<sup>22</sup> as noted in his table 4, support such an hypothesis. He found that 3% agar was penetrated more rapidly than 2 or 1%, but that 2% was penetrated slightly less rapidly than 1%.

The favorable influence of the absence of oxygen is supplemented by the thin consistency of the agar permitting ready extension of the bacteria through it by development or motility. They thus find it possible to utilize all the nutriment present in a way that is impossible in solid mediums.

On the second point, we find in the literature of the cultivation of spirochetes some strikingly suggestive statements. Especially illuminating is the observation of Noguchi:<sup>24</sup> "It is very important to employ samples of ascitic fluids which contain no bile, but which form a loose fibrin in the culture tube, for many specimens are unsuitable just because they contain too much bile or do not cause the formation of fibrin when mixed with the fresh tissue in the culture tube."

Zinsser, Hopkins and Ruth Gilbert,<sup>25</sup> in attempting to find a more simple technic for the cultivation of *Treponema pallidum*, found that clear fluid serum mediums are unsuitable, and it was necessary to coagulate the serum partially as Schereschewsky<sup>26</sup> had done originally. They say

It is interesting to note that we were not able to grow the culture on horse serum without tissue unless the serum had been heated and gelatinized. When the serum had been heated in this way, our strain grew both with and without the addition of tissue, and not only on the horse serum but also on similarly prepared sheep and beef serum.

These observations, together with those of our own noted in the foregoing, suggest that we are dealing with a fundamental principle in the artificial cultivation of disease-producing organisms. Until the work of Noguchi, efforts of bacteriologists had been directed almost exclusively to the production of artificial culture mediums identical chemically with the particular tissues and tissue fluids most favorable to the bacterium in question, as exhibited by the part of the body in which it most frequently found parasitic residence. Bacteria are classified into facultative and strict parasites according to their ability to grow in liquid mediums or on solid mediums which may bear no physical resemblance to their tissue habitat. We consider the colon bacillus practically a saprophyte because we can cultivate it with so little difficulty—in the body, it grows in the contents of the intestine. The spirochetes and some of the gram-negative cocci, and doubtless other bacteria, seem to require formed substances resembling tissues or cells to which they may cling or on which they may settle while taking their nourishment from surrounding fluids. We may find that for certain bacteria, when placed in mediums physically similar to the tissues, chemical composition of the fluids will be of as little consequence as physical conditions are to those bacteria of which the colon bacillus is the type.

<sup>24</sup> Jour. Exp. Med., 1912, 16, p. 199.

<sup>25</sup> Ibid., 1915, 21, p. 213.

<sup>26</sup> Deutsch. med. Wchnschr., 1909, 35, p. 835.

## SUMMARY

In agar culture mediums containing 0.1% agar, the gel, composed of colloidal particles in a state of equilibrium, resists the penetration of oxygen and consequently offers excellent conditions for the development of anaerobic bacteria. It is therefore suggested as a medium for the primary cultivation of specimens suspected of containing anaerobic bacteria, and for the more convenient study of pure cultures especially with regard to their physiologic relations. The broth above the agar is naturally an excellent culture medium for the aerobes which develop well in ordinary mediums; in addition, the presence of the agar underlying or suspended through the broth makes it, like water of condensation, a good medium for certain others, the gonococcus and meningococcus, for instance, which grow with difficulty or not at all in ordinary mediums.

Culture mediums containing low percentages of agar, since they offer at the same time suitable conditions for the development of both aerobic and anaerobic bacteria, should be valuable for bacteria requiring partial oxygen tension and for the detection of contamination in such substances as the so-called biologic products. The medium should be especially valuable for this purpose since the disinfectant generally used for the preservation of biologic products is diluted and removed from direct contact with any bacteria that may be present just as the metabolic products of the gonococcus, for instance, are diluted and removed.

It is suggested that this medium, composed of a definite stroma of agar containing droplets of broth, with a supernatant reservoir of broth, bears a certain important physical resemblance to the tissues and their fluids. With this basic medium, chemical "differentiation" may be accomplished, if necessary, through the addition of special substances. It therefore presents advantages over ordinary mediums and offers possibilities for the development of our knowledge of groups of bacteria whose study has heretofore been surrounded by great difficulties.

Not least among the points in favor of the medium is its great simplicity in that it contains only the ingredients of our most commonly used culture substrate—ordinary nutrient agar.

# THE EFFECT OF THE DIPHTHERIA TOXIN ON THE BLOOD AND HEMOPOIETIC ORGANS OF RABBITS

WITH PLATE

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Although many details have been added to the knowledge of diphtheria toxin since its discovery, still its effect on the blood is not fully understood.

Bouchut and Dubrisay<sup>1</sup> noted leukocytosis as usual in diphtheria, and later the former<sup>2</sup> found that the number of leukocytes increases with the severity of the disease and decreases on improvement. After a careful study of leukocytic reaction in animals and in clinical cases, Gabritchewsky<sup>3</sup> concluded that a progressive leukocytosis signifies a bad prognosis in diphtheria, and this is in accord with the results of Nicolas and Courmont,<sup>4</sup> Schlesinger,<sup>5</sup> and Morse,<sup>6</sup> but contrary to those of Besredka,<sup>7</sup> who observed that leukocytosis has no relation to the severity of the disease, but that a decrease in the number of polymorphonuclear leukocytes, together with the presence of "intermediate cells," means an unfavorable prognosis. Furthermore, his results showed that in the animals inoculated with a massive dose of diphtheria toxin the number of leukocytes after reaching its maximum gradually declined till the death of the animal. Ewing<sup>8</sup> stated that in fatal cases the number of leukocytes may be steadily increased or decreased, or there may be no leukocytosis. Arneth<sup>9</sup> found leukopenia in one fatal case, and also recorded the occurrence of myelocytes in two fatal cases. As to the presence of myelocytes in the blood of diphtheria patients, Engel<sup>10</sup> was the first to call attention to the fact that the number of myelocytes increases as the disease advances. He found from 3 to 11 % of myelocytes in the fatal cases.

Gabritchewsky<sup>3</sup> demonstrated necrosis of leukocytes in rabbits inoculated with diphtheria bacilli in the anterior chamber of the eye, the leukocytes appearing in large numbers at 8 hours and reaching its maximum at 24 hours after inoculation. Metchnikoff<sup>8</sup> observed the same phenomena and concluded that phagocytosis by leukocytes in diphtheria plays a minor part. Ewing<sup>8</sup> claimed that there is a marked leukocytic degeneration as evidenced by the presence of leukocytic shadows of Klein and deficiency in the granules and chromatin of polymorphonuclear leukocytes; the latter condition in one case

Received for publication June 6, 1921.

<sup>1</sup> Compt. rend. Acad. d. sc., 1877, 85, p. 158.

<sup>2</sup> Gaz. d. hôp., 1879, 52, p. 153.

<sup>3</sup> Ann de l'Inst. Pasteur, 1894, 8, p. 673.

<sup>4</sup> Arch. de méd. expér., 1897, 9, p. 737.

<sup>5</sup> Arch. f. Kinderheilk., 1896, 19, p. 378; 1900, 30, p. 233.

<sup>6</sup> Boston Med. & Surg. Jour., 1895, 132, pp. 228, 252.

<sup>7</sup> Ann. de l'Inst. Pasteur, 1898, 12, p. 305.

<sup>8</sup> Clin. Path. of the Blood, 1903, p. 290.

<sup>9</sup> Die neutrophilen weissen Blutkörperchen, 1904, p. 79.

<sup>10</sup> Deutsch. med. Wchnschr., 1897, 23, pp. 118 and 137.



was associated with leukopenia. He also detected an increased acidophile tendency in the neutrophile granules. Welch and Flexner<sup>11</sup> noted karyorrhexis of leukocytes in the lungs and liver of their animals. Barbacci,<sup>12</sup> studying the changes of the spleen, lymph nodes and liver in diphtheria, found chromatin materials in the splenic pulp, which were supposed to be derived from degenerated leukocytes. He called diphtheria toxin karyolytic and said that it works not only on the fixed lymphatic elements, but also on the mobile the leukocytes of the blood. Schurmann<sup>13</sup> induced karyorrhexis of leukocytes in vitro by diphtheria toxin.

Councilmann, Mallory and Pearce<sup>14</sup> observed a hyperplasia of marrow as a usual occurrence in diphtheria, and in one case they found an area of hemorrhage and necrosis. Roger and Josus<sup>14</sup> studied the effect of diphtheria toxin and antitoxin on the marrow; they found that the former produces a proliferation of large and medium cells and the latter that of small cells. Trambusti<sup>14</sup> called attention to the fact that the reaction of marrow depends on the amount of toxin, large doses causing a paralytic action and a small one hyperplasia. Morse<sup>15</sup> described a hyperplasia of small cells with small round and deeply staining nuclei. Dickson<sup>15</sup> found vacuolation and karyorrhexis in marrow cells to be induced by diphtheria toxin.

The changes in the spleen and lymph nodes in diphtheria have been thoroughly described by various authors. However, the question of the large cells in the center of malpighian bodies still remains open. Bizzozero<sup>12</sup> was the first to describe the large cells with pale, round or oval nuclei that sometimes carry globular bodies within the cytoplasm. Oertel<sup>16</sup> emphasized their epithelial character. Ziegler<sup>17</sup> considered them as swollen cells of the reticulum and Ribbert<sup>18</sup> thought that they are derived from the endothelium. Councilmann, Mallory and Pearce<sup>14</sup> found these cells usually in the early stages of infection and regarded them as similar to the large cells in tubercle and as phagocytic. Waschkevitch<sup>19</sup> claimed that they are degenerated leukocytes which have wandered into the malpighian bodies.

The general technic applied in this study is the same as described in my previous article<sup>20</sup> on the effect of hemolytic streptococci on the blood and hemopoietic organs of rabbits. The diphtheria toxin tested 0.012 c c MLD. The animals were inoculated intravenously with various amounts of diphtheria toxin in 1 c c of salt solution.

The following tables serve to illustrate the changes in the leukocytes in vivo and vitro.

<sup>11</sup> Bull. Johns Hopkins Hosp., 1896, 2, p. 107.

<sup>12</sup> Centralbl. f. Allg. Path., 1896, 7, p. 321.

<sup>13</sup> Ibid., 1910, 21, p. 337.

<sup>14</sup> Jour. Bost. Soc. Med. Sc., 1900, 5, p. 139.

<sup>15</sup> The Bone-Marrow, 1908.

<sup>16</sup> Die Pathologie der epidemischen Diphtheritis, München, 1887.

<sup>17</sup> Text-Book of Spec. Path. Anat. (Eng.), 1897, sec. 3, p. 110.

<sup>18</sup> Lehrbuch der Path. Anat., 1896.

<sup>19</sup> Virchows Arch., 1900, 159, p. 137.

<sup>20</sup> Jour. Infect. Dis., 1921, 29, p. 141.

*Experiment 1.*—A rabbit, weighing 3,004 gm., received 0.6 cc of diphtheria toxin. The animal appeared very sick for 2 hours after inoculation and died within 20 hours.

TABLE 1  
FINDINGS IN EXPER. 1

	Total Leuko- cytes	Percentage							
		Ampho- phile	Baso- phile	Eosino- phile	Lym- pho- cytes	Large Mono- nuclear	Transi- tional	Myelo- cytes	Degen- erated Cells
Before inocula- tion.....	12,000	29.5	1.5	0	67.5	1.5	0	0	0
½ hour after.....	10,000	28.5	3.0	0.5	67.5	0.5	0	0	0
1 hour after.....	9,000	70.5	1.0	3.0	24.5	1.0	0	0	0
2 hours after.....	6,550	69.5	2.5	0	26.0	2.0	0	0	32
4 hours after.....	3,550	59.0	4.5	0	35.5	1.0	0	0	45
6 hours after....	5,020	12.0	0	0.5	80.0	2.5	0	5	46
16 hours after....	4,850	24.5	3.5	0.5	61.0	2.5	0	8	62

*Experiment 2.*—A rabbit, weighing 2,750 gm., received 0.4 cc of diphtheria toxin and died within 24 hours.

TABLE 2  
FINDINGS IN EXPER. 2

	Total Leuko- cytes	Percentage							
		Ampho- phile	Baso- phile	Eosino- phile	Lym- pho- cytes	Large Mono- nuclear	Transi- tional	Myelo- cytes	Degen- erated Cells
Before inocula- tion.....	14,300	35.5	3.5	0.5	60.0	0.5	0	0	0
½ hour after.....	12,500	46.5	1.0	0.5	51.5	0.5	0	0	0
1 hour after.....	11,950	56.0	3.0	0	39.0	2.0	0	0	0
2 hours after.....	12,050	54.5	3.5	0	40.0	2.0	0	0	26
4 hours after.....	9,675	52.0	4.5	0	41.5	2.0	0	0	19
6 hours after.....	8,450	36.0	3.0	0.5	26.0	4.0	0	30.5	21
16 hours after....	7,650	38.0	3.5	0	29.0	7.0	0	22.5	47

*Experiment 3.*—A rabbit, weighing 2,875 gm., received 0.1 cc of diphtheria toxin and also died within 24 hours.

TABLE 3  
FINDINGS IN EXPER. 3

	Total Leuko- cytes	Percentage							
		Ampho- phile	Baso- phile	Eosino- phile	Lym- pho- cytes	Large Mono- nuclear	Transi- tional	Myelo- cytes	Degen- erated Cells
Before inocula- tion.....	10,725	42.5	5.0	0	51.5	1.0	0	0	0
½ hour after.....	10,950	45.0	4.0	0	50.0	1.0	0	0	0
1 hour after.....	10,650	41.5	4.5	0	49.0	5.0	0	0	0
2 hours after....	10,600	46.5	3.0	0	44.5	6.0	0	0	0
4 hours after....	10,675	57.0	2.5	0	40.5	0	0	0	0
6 hours after....	8,550	41.5	4.0	0	32.5	0	0	12	13
16 hours after....	10,825	39.0	2.5	0	24.5	0	0	34	18

*Experiment 4.*—Rabbit A, weighing 2,415 gm., received 0.05 c c of diphtheria toxin and died about 30 hours after inoculation.

Rabbit B, weighing 2,600 gm., received 0.05 c c of diphtheria toxin; and died in about 48 hours.

TABLE 4  
FINDINGS IN EXPER. 4

	Total Leuko- cytes	Percentage							
		Ampho- phile	Baso- phile	Eosino- phile	Lym- pho- cytes	Large Mono- nuclear	Transi- tional	Myelo- cytes	Degen- erated Cells
Rabbit A:									
Before inocula- tion.....	12,925	36.5	4.5	0	54.0	5.0	0	0	0
½ hour after...	12,850	40.0	3.0	1.0	52.0	4.0	0	0	0
1 hour after...	13,250	38.5	3.5	1.0	53.5	3.5	0	0	0
2 hours after...	12,950	39.0	3.5	0	54.0	3.5	0	0	0
4 hours after...	13,000	34.5	2.0	0.5	59.0	4.0	0	0	0
6 hours after...	9,025	45.5	3.5	0	47.0	4.0	0	0	0
16 hours after...	12,050	46.0	3.0	1.0	48.0	2.0	0	0	0
20 hours after...	13,750	49.5	4.5	0.5	39.0	6.5	0	0	0
24 hours after...	41,800	32.5	1.5	0	61.0	5.0	0	0	23
Rabbit B:									
Before inocula- tion.....	12,025	26.0	1.0	0.5	72.0	0.5	0	0	0
½ hour after...	12,450	31.0	2.5	0	64.0	2.5	0	0	0
1 hour after...	11,950	30.0	3.0	0.5	66.0	0.5	0	0	0
2 hours after...	9,675	45.5	5.0	0.5	49.0	0	0	0	0
4 hours after...	10,950	48.0	1.5	1.5	49.0	0	0	0	0
6 hours after...	12,000	52.0	3.0	0	37.0	8.0	0	0	0
8 hours after...	14,250	58.5	4.0	0.5	29.0	8.0	0	0	0
24 hours after...	53,750	69.0	0.5	0	30.5	0	0	0	32
30 hours after...	34,500	51.5	2.5	0	18.5	2.5	0	25	46

*Experiment 5.*—A rabbit, weighing 2,456 gm., received 0.01 c c of diphtheria toxin and died on the fourth day after inoculation.

TABLE 5  
FINDINGS IN EXPER. 5

	Total Leuko- cytes	Percentage							
		Ampho- phile	Baso- phile	Eosino- phile	Lym- pho- cytes	Large Mono- nuclear	Transi- tional	Myelo- cytes	Degen- erated Cells
Before inocula- tion.....	14,425	37.5	2.0	0	60.0	0.5	0	0	0
½ hour after....	9,975	31.5	1.5	0	66.0	1.0	0	0	0
1 hour after.....	8,125	37.5	3.0	1.0	56.5	2.0	0	0	0
2 hours after....	9,675	33.0	3.0	0	64.0	0	0	0	0
4 hours after....	8,225	41.0	3.0	0	54.5	1.5	0	0	0
6 hours after....	12,175	42.0	0.5	0	56.5	1.0	0	0	0
24 hours after....	12,650	50.0	3.5	0	46.5	0	0	0	0
30 hours after....	14,450	56.0	2.5	0	39.5	2.0	0	0	0
48 hours after....	10,675	61.5	3.0	0	34.0	1.5	0	0	0
54 hours after....	10,625	59.5	3.0	0	32.5	5.0	0	0	0
72 hours after....	24,375	38.5	4.0	0.5	34.5	9.5	0	13	0

*Experiment 6.*—Rabbit A, weighing 2,350 gm., was immunized with 1,500 units of diphtheria antitoxin 12 hours before receiving 0.1 c c of diphtheria toxin. The animal survived, but both hind legs were paralyzed on the fourth day of the experiment.

Rabbit B, weighing 2,650 gm., was immunized with 1,500 units of diphtheria antitoxin 12 hours before receiving 0.05 c c of diphtheria toxin. The animal survived.

TABLE 6  
FINDINGS IN EXPER. 6

	Total Leuko- cytes	Percentage							
		Ampho- phile	Baso- phile	Eosino- phile	Lym- pho- cytes	Large Mono- nuclear	Transi- tional	Myelo- cytes	Degen- erated Cells
Rabbit A:									
Before inocula- tion.....	10,900	47.0	5.0	0.5	47.0	0.5	0	0	0
½ hour after...	9,200	49.5	4.0	0	44.0	2.5	0	0	0
1 hour after...	8,725	74.0	3.0	1.0	20.0	2.0	0	0	0
2 hours after...	9,800	52.0	2.5	0.5	42.5	2.5	0	0	15
4 hours after...	12,700	61.0	3.5	0	32.0	3.5	0	0	14
6 hours after...	10,475	59.0	2.0	0	37.0	2.0	0	0	0
24 hours after...	9,225	46.0	3.5	1.0	45.0	4.5	0	0	0
48 hours after...	9,675	47.5	4.0	0	44.0	4.5	0	0	0
72 hours after...	13,675	51.5	3.5	1.5	36.0	7.5	0	0	0
96 hours after...	9,850	46.0	3.0	0.5	48.0	2.5	0	0	0
Rabbit B:									
Before inocula- tion.....	9,000	37.0	6.5	0.5	56.0	0	0	0	0
½ hour after...	8,725	44.5	3.0	0	52.5	0	0	0	9
1 hour after...	9,250	51.0	4.5	1.5	49.0	4.0	0	0	0
2 hours after...	10,475	51.0	6.5	0.5	34.0	8.0	0	0	0
4 hours after...	12,875	48.0	4.0	2.0	37.0	9.0	0	0	0
6 hours after...	11,925	39.0	4.0	0.5	49.5	7.0	0	0	0
24 hours after...	10,425	40.0	4.0	1.5	47.5	7.0	0	0	0
48 hours after...	12,225	52.0	4.0	0.5	35.0	8.5	0	0	0
72 hours after...	9,875	42.0	3.0	0	46.0	9.0	0	0	0
96 hours after...	9,650	46.5	4.5	0	43.0	6.0	0	0	0

*Experiment 7.*—The leukocytic exudate was suspended in various amounts of diphtheria toxin incubated at 37 C. to test its injurious effect and also to see whether this effect can be neutralized by the antitoxin. Smears were made every half hour and stained with methylene blue. The results are indicated in table 7.

TABLE 7  
FINDINGS IN EXPER. 7

Leukocytes	Toxin	Salt Solution	Results		
			½ Hour	1 Hour	1½ Hours
0.25 c c	0.1 c c	0.65 c c	+	+	+
0.25 c c	0.075 c c	0.675 c c	+	+	+
0.25 c c	0.05 c c	0.7 c c	0	+	+
0.25 c c	0.025 c c	0.725 c c	0	+	+
0.25 c c	0.01 c c	0.74 c c	0	+	+
0.25 c c	0.0075 c c	0.7425 c c	0	0	±
0.25 c c	0	0.75 c c	0	0	0

TABLE 7.—Continued

Leukocytes	Toxin	Antitoxin	Salt Solution	Results		
				½ Hour	1 Hour	1½ Hours
0.25 c c	0.1 c c	0.1 c c	0.55 c c	+	+	+
0.25 c c	0.075 c c	0.1 c c	0.575 c c	+	+	+
0.25 c c	0.05 c c	0.1 c c	0.6 c c	0	0	±
0.25 c c	0.025 c c	0.1 c c	0.625 c c	0	0	0
0.25 c c	0.01 c c	0.1 c c	0.64 c c	0	0	0
0.25 c c	0.0075 c c	0.1 c c	0.6425 c c	0	0	0
0.25 c c	0	0.1 c c	0.65 c c	0	0	0

+, a definite degeneration of leukocytes; ±, doubtful; 0, negative.

It is evident that the amount of diphtheria toxin is an important factor in determining the leukocytic reaction. A massive dose as a rule leads to leukopenia, which prevails throughout the course of the intoxication. The blood smears now show marked retrogressive changes in the leukocytes, and the marrow after death presents no evidence of hyperplasia, the marrow cells being more or less degenerated. It seems safe to say that this leukopenia is due to the directly injurious effect of diphtheria toxin on the marrow and the leukocytes in the circulation. It has been thought that when leukopenia occurs after an intravenous injection it may be brought about by an irregular distribution of the leukocytes in internal organs. In my work the organs taken from the animals in the stage of leukopenia in certain cases presented some evidence of accumulation of leukocytes and in others no difference in comparison with control specimens so far as concerns the number of leukocytes. In immunized animals 0.05 c c of diphtheria toxin produced no appreciable decrease in the number of leukocytes, whereas in the nonimmunized animals the same quantity of toxin caused leukopenia about 6 hours after inoculation. This difference indicates that the antitoxin is able to neutralize the destructive effect of diphtheria toxin on the leukocytes. In experiment 4 there was a progressive leukocytosis with a fatal termination. This seems to be in accord with the results of Gabritchewsky and others but, on the other hand, it must be understood that the amount of toxin used in this experiment was comparatively small and such an amount of toxin may be detrimental to the life of the animal and yet not so destructive to the marrow and the leukocytes in the circulation that leukopenia arises. Myelocytes were found in all the rabbits injected with toxin. In experiment 2 the number of myelocytes was so large that a differential count would remind one of an acute myeloid leukemia, but the total number of leukocytes and the depletion of marrow rather speak

against such deduction. It is difficult to account for the entry of normal myelocytes into the circulation when the marrow is undergoing marked degeneration, but in almost all cases I found a few cells that still maintained their integrity, and these cells, which I believe escaped the toxic effect, subsequently would migrate into the circulation to meet the demand.

The diphtheria toxin acts on both the nucleus and cytoplasm of all the types of leukocytes, and the degree of the leukocytic degeneration largely depends on the quantity of toxin. Furthermore, antitoxin apparently is able to check this injurious effect as illustrated by experiments 6 and 7. In severe cases the nuclei of amphophiles were swollen and pale or became excessively faint. Sometimes they increased in lobulation and stained well with basic dyes. The pyknosis was encountered rarely. The granules of amphophiles were swollen and few in number, and scattered on a pale pink cytoplasm. The nuclei of lymphocytes usually appeared normal, but in acute cases they also underwent the same changes as those of amphophiles. Their cytoplasm was occasionally fragmented or converted into dark spherical bodies 3-4 in number attached to the nucleus. The so-called basket-shaped cells were seen at times.

The changes in the marrow vary with the amount of diphtheria toxin and somewhat also with the duration of action. In addition to the hyperplasia which has been regarded as a usual occurrence in all acute infections, there were various degenerative changes which occurred in a marked degree in the animals inoculated with a massive dose of diphtheria toxin. The diphtheria toxin chiefly affected the nuclei, but the cytoplasm was also more or less involved. The nuclei of myelocytes usually appeared to be swollen, and the chromatin became aggregated into spherical bodies around the perinuclear membrane and stained strongly with basic dyes. This condition probably indicates an early stage of karyorrhexis. Occasionally these spherical bodies were seen in groups here and there in the areas of degeneration. The cytoplasm at first appeared granular and stained well with eosin; later it became swollen and vacuolated and also its affinity for eosin seemed to be lost. The marrow as a rule underwent a hyperplasia in the early stages of experiments regardless of the amount of diphtheria toxin. In experiment 4 one of the animals showed a hyperplasia of small cells with small round and deeply staining nuclei. Of these cells some possessed a large quantity of cytoplasm with an uneven edge,

some had a narrow pink rim around the nucleus and still others presented only a deeply staining nucleus. By the smear method it was found that the majority of the cells were of the lymphoid type, as described by Councilmann, Mallory and Pearce. At this point it is worthy of mention that this animal had lymphocytosis shortly before the death. Megalokaryocytes were few in number and all more or less degenerated, but in the immunized animals they were frequently encountered. The occurrence of phagocytes seemed to have no relation to the stage of inoculation, which is contrary to Dickson's observation, as he found them more numerous in the early stages. Hemorrhage and congestion were of frequent occurrence.

The constant change in the spleen was karyorrhexis, usually in the malpighian bodies. The nuclear fragments in most cases were scattered throughout the lesion and sometimes contained in phagocytes which had a large and pale nucleus and a pink granular cytoplasm with an irregular edge. Occasionally the chromatin of lymphocytes in the malpighian bodies was seen as dark spherical bodies around the perinuclear membrane. The nuclei of the lymphocytes in the splenic pulp and of the reticular cells in the early stage were shrunken and dark, and they appeared to be similar to polymorphonuclear leukocytes. This is probably an early stage of degeneration prior to karyorrhexis. Around these cells dark spherical bodies of various size were encountered also. The so-called epithelioid cells usually occurred in the spleen taken from the immunized animals or from the animals killed in the early stage. These cells were usually definitely marked out and arranged in rows and occasionally scattered among the lymphocytes. The nuclei were large, pale and oval or round, occasionally with a nucleolus which stained pink, and the cytoplasm stained poorly with eosin. None of these cells presented any evidence of phagocytic activity. I assume that they were lymphocytes and reticular cells which were undergoing a retrogressive change. The congestion and hemorrhage occurred nearly in all cases but the degree of the latter varied with the severity of infection. Hyaline degeneration was demonstrated in the mild cases.

The changes in the lymph nodes were practically the same as those in the spleen and for this reason I shall deal with only the conditions which were not found in the latter. In experiment 5 the sinuses of the lymph nodes were distended either with yellowish granular coagulum or with phagocytes and erythrocytes. In this case the lymph node also showed a certain degree of hyperplasia and degeneration.

The observations on the effect of diphtheria toxin on the erythrocytes revealed nothing important. In one case there was a slight decrease in the number of erythrocytes and the percentage of hemoglobin with an occasional appearance of normoblasts and crenated erythrocytes.

#### CONCLUSIONS

Diphtheria toxin is destructive to the leukocytes *in vivo* as well as *in vitro*.

In massive doses it usually produces a retrogressive change in the hemopoietic organs.

Leukopenia in animals inoculated with a massive dose of diphtheria toxin is in all probability due to the degeneration of the hemopoietic organs and the leukocytes in the circulation caused by diphtheria toxin.

The so-called epithelioid cells in the malpighian bodies seem to be the degenerated lymphocytes and reticular cells.

Antitoxin in a proper portion is able to neutralize the destructive effect of diphtheria toxin on the hemopoietic organs and on the leukocytes *in vivo* and *in vitro*.



# PLATE 1

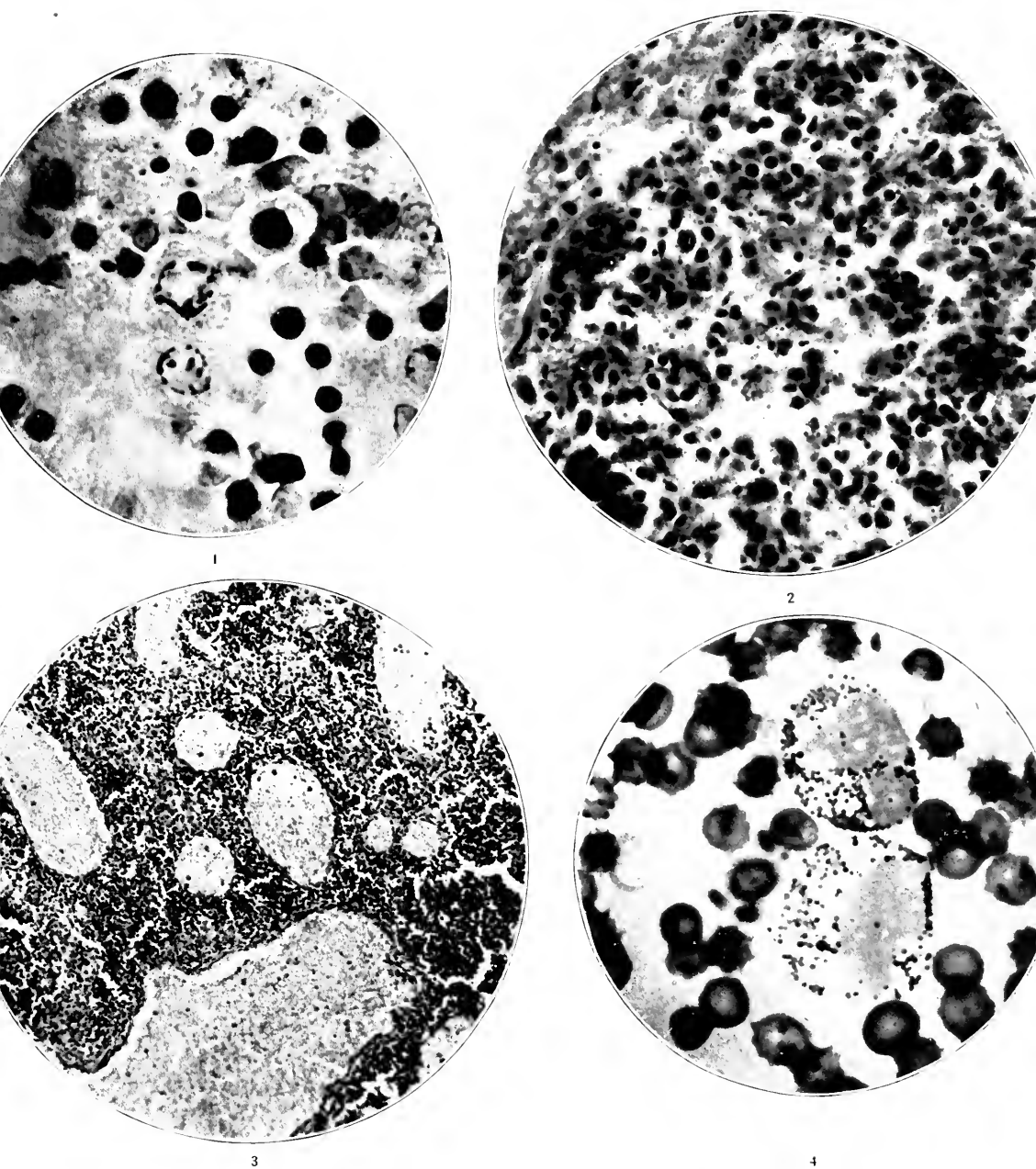


Fig. 1 (Exper. 2).—Marrow showing an early stage of karyorrhexis of myelocytes, and also a large number of cells with round and deeply staining nuclei; slightly reduced from a photomicrograph. X 1200.

Fig. 2 (Exper. 4).—Karyorrhexis in malpighian body; slightly reduced from a photomicrograph. X 600.

Fig. 3 (Exper. 5).—Lymph node showing karyorrhexis, hyperplasia and a marked distention of sinuses; slightly reduced from a photomicrograph. X 170.

Fig. 4 (Exper. 3).—A blood smear about 2 hours before the death of the animal showing karyolysis of myelocytes. To their left a degenerated lymphocyte with 3 dark spherical bodies; slightly reduced from a photomicrograph. X 1200.



# AN EXPERIMENTAL STUDY OF SALINE AND LIPOID TYPHOID VACCINES IN RESPECT TO ANTI- GENIC AND IMMUNIZING VALUE

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Mineral and vegetable oils have had a varied use in the medical world, but the possibilities of employing them as a medium for the suspension of vaccines were not conceived until the stress of the late war showed that the modern methods of prophylaxis were not wholly adequate and very time-consuming. Wassermann<sup>1</sup> had made a staphylococcus vaccine in the form of an unguentum with the idea of avoiding hypodermic injection. Zeuner<sup>2</sup> used soap solution in the preparation of tuberculin to get a more complete solution rather than increased absorption. Le Moignic and Sezary<sup>3</sup> showed that it was possible to produce a high hemolytic serum by injecting red cells in oil as well as in salt solution. The oil suspension gave slow absorption and acted as a detoxifying agent.

Le Moignic and Pinoy<sup>4</sup> grasped the possibilities of lipovaccines in prophylaxis and a number of other investigators worked on this subject at about the same time. Le Moignic and Pinoy employed liquid petrolatum and lanolin as a base for the vaccines but later substituted vegetable oils. Archard and Foix<sup>5</sup> gave similar reports on work with olive oil as a base but noted one disadvantage, namely, that oil heated to too high a temperature during sterilization may cause abscesses. Le Moignic and Sezary<sup>6</sup> and Le Moignic and Gautrelet<sup>7</sup> claimed that the incorporation of heated and ground cultures in oil raises the vaccinating property and lowers the toxic action because the oil exerts a retarding action on both toxin liberation and absorption. Lipovaccine, being less toxic, can be injected in large amounts without any inconvenience in a single dose. Le Moignic and Gautrelet introduced large doses of lipovaccine directly into the veins without any ill effects.

The chief advantages, claimed by the French, from the use of lipovaccines may be summarized as: (1) a diminution of local and systemic reactions, (2) proper immunization produced by a single injection, (3) the persistence in the individual of a focus from which the immunization proceeds for many months resulting in a prolonged period of immunity, (4) a detoxicating effect of certain lipoids incorporated in the vaccine, and (5) the prevention of autolysis and deterioration of the vaccine. These claims are sufficient to warrant a thorough study of the lipovaccines.

Whitmore, Fennel and Peterson,<sup>7</sup> therefore, undertook a study of lipovaccines in typhoid, meningococcus and dysentery prophylaxis. They reported that

Received for publication June 6, 1921.

<sup>1</sup> Cited in Jour. Am. Med. Assn., 1918, 70, p. 428.

<sup>2</sup> Compt. rend Soc. de biol., 1917, 80, p. 1797.

<sup>3</sup> Ibid., 1916, 79, p. 201 and 352.

<sup>4</sup> Ibid., p. 209.

<sup>5</sup> Bull. de l'Inst. Pasteur, 1918, 16, p. 263.

<sup>6</sup> Ibid., p. 265.

<sup>7</sup> Jour. Am. Med. Assn., 1918, 70, pp. 427 and 902.

they could give in dysentery vaccine, 3,000 million "Shiga," 3,200 million Bacillus "Y" and 2,200 million Flexner organisms in oil without marked local or general reaction. They claimed to find the production of agglutinins, precipitins and bacteriolysins in the blood of vaccinated animals and men as well as evidence of alexin-fixation. They further stated that the agglutination titer of rabbits immunized with a single dose of lipovaccine compares favorably with that obtained with three doses of aqueous vaccine and that protective experiments on guinea-pigs indicates a degree of protection equal to that obtained with aqueous vaccine.

This last statement was not fully verified by experiment and it therefore suggested itself as worth investigating. Our plan was to compare the agglutinin production in rabbits immunized with typhoid lipovaccine and saline vaccine, respectively, and to test the degree of protection afforded against the typhoid carrier state in rabbits, according to the method of Gay and Claypole.<sup>8</sup>

*Vaccine Preparation.*—Whitmore, Fennel and Peterson<sup>7</sup> prepared their lipovaccine in a complicated manner, involving the drying of a 24-hour growth of the organism in an oven at 53 C. through which a current of sterile air was passed continuously; second, they ascertained the dry weight and ground the preparation in sterile anhydrous lanolin from 6-8 hours, and then ground it in olive oil for 24 hours. Finally, the vaccine was heated for 1 hour at 53 C. and sterility tests were made.

Pfeiffer and Bessau<sup>9</sup> found that heating a saline suspended vaccine extracts the antigenic substances from the bacteria rapidly and thoroughly. This might be true in the case of oil suspensions. The effect of heat on the lipovaccine has been made the subject of investigation by Lewis and Dodge<sup>10</sup> who found that to sterilize a lipovaccine successfully, it must be subjected to 130 C. for 3 hours or 120 C. for 12 hours in an electric oven. Loeffler<sup>11</sup> stated that the application of dry heat to bacteria kills without destroying the antigenic properties. Lewis and Dodge<sup>10</sup> found that this varies with the organism since in applying an intermediate temperature of 130 C. for 3 hours the antigenic properties are not destroyed in the pneumococcus vaccine but are in a typhoid vaccine.

Rosenow and Osterberg<sup>12</sup> shortened the process by killing the bacteria by a watery solution of an antiseptic, then formed an emulsion with oil and removed the water by vacuum distillation at a low temperature. The oil emulsion served to prevent the bacteria from clumping and so eliminated the time-consuming grinding process.

Our own method of preparation of saline and lipovaccine was as follows:

Blake bottles containing 2 % meat infusion agar of a titer of + 1 were seeded with a 24-hour broth culture of *B. typhosus* 3. After 24 hours, the growth was washed off in 25 cc of sterile 0.85 % salt solution. This suspension was precipitated with absolute alcohol, centrifugalized and dried to a constant weight over sulphuric acid in vacuo.

<sup>8</sup> Arch. Int. Med., 1913, 12, p. 613.

<sup>9</sup> Centralbl. f. Bakteriöl., I, O., 1912, 64, p. 172.

<sup>10</sup> J. Exper. Med., 1920, 31, p. 169.

<sup>11</sup> Deutsch. med. Wehnschr., 1913, 39, p. 1025.

<sup>12</sup> Jour. Am. Med. Assn., 1919, 73, p. 87.

The material was ground for 2 hours and separated into two portions by weight. Portion A, which was one-third the dry weight of portion B, was suspended in 0.5 % phenolated salt solution in sufficient volume to make each c c contain  $\frac{1}{6}$  mg. or 1,333 million bacteria per mg. accepting Wilson and Dickson's<sup>13</sup> standard of 8,000 million per mg. of dried typhoid bacilli. Portion B was three times the dry weight of portion A and was suspended in 0.5 % phenolated olive oil containing  $\frac{1}{10}$  volume of sterile anhydrous lanolin. Suspension B contained 0.5 mg. or 4,000 million bacteria per c c. Sterility tests were made. The technic of Whitmore, Fennel and Peterson was in the main adhered to, with the exception of drying the bacteria in an oven at 53 C. or freezing, and the grinding period was decreased from 8 hours in lanolin and 24 hours in oil to 2 hours of grinding in the dry form and then suspending in oil or saline.

*Immunization of rabbits.*—In all, 30 rabbits, ranging from 1,600 to 3,000 gm. in weight, were vaccinated. Those vaccinated with saline vaccine received subcutaneously 1 c c or  $\frac{1}{6}$  mg. dry weight of typhoid vaccine on 3 alternate days. At the time of the third inoculation, the remaining rabbits were given, subcutaneously, one dose of 1 c c or 0.5 mg. of lipovaccine. A record of weight kept during the immunization period showed that vaccination usually produced no ill effects or loss in weight. Of the 30 animals vaccinated, 8 died before inoculation with a living culture of *B. typhosus*. In no case could the cause of death be traced to any bacterial infection.

*Antibody Production.*—A. Agglutinins: The agglutinin production was observed over 4, 8 and 12 week periods. Each rabbit, at the expiration of its respective period, received an infecting dose of *B. typhosus* in an attempt to determine the protection afforded, according to the method of Gay and Claypole.<sup>8</sup> The lipoid vaccine, irrespective of the time factor, does not usually stimulate agglutinin production. Transitory and weak agglutinins in a dilution of 1:20 were found. Following saline vaccination, agglutinins appear in the blood stream in dilutions of 1:160 and 1:1,280 depending on the factors of time and individual response. The tests have been carried out at 37 C. and 56 C. with the same result. The agglutinin titer, in the serums of all rabbits, regardless of treatment, was greatly increased after infection and showed a positive Widal reaction in dilutions of 1:1,280 or 1:2,560

<sup>13</sup> J. Hyg., 1912, 12, p. 49.

with regularity. This titer did not decrease during 6 weeks, which was the limit of our observation.

The abrupt and rapid rise of agglutinins from zero to 1:1,280 in animals vaccinated with lipovaccine does not differ from the response of the normal rabbit. Agglutinins appear a day earlier in the vaccinated animal, but do not exceed or reach the final titer any earlier.

B. Alexin-Fixation: Since we were unable to demonstrate any agglutinins in the serums of animals immunized with lipoid typhoid vaccine, it was suggested that the antibody content of this serum might be shown by the alexin-fixation reaction of Bordet and Gengou. Tribondeau<sup>14</sup> stated that the alexin-fixing substances appear in the serum following lipovaccines about the same time as the agglutinins and disappear about the end of the second month. The serum from about one half of our rabbits were tested and no fixation was obtained in the oil vaccinated rabbits previous to infection with *B. typhosus* and

TABLE 1  
MEAN AGGLUTININ TITER

Treatment	No. of Animals	Mean Agglutinin Titer Before Infection	Mean Agglutinin Titer After Infection	Result on Infection
Saline vaccine .....	10	1-160	1-2560	30% carriers
Oil vaccine .....	12	0	1-2560	33½% carriers
Normal .....	10	0	1-2560	80% carriers

the serum of saline vaccinated rabbits gave only slight fixation. Medlar<sup>15</sup> notes that there is only partial fixation after vaccination with lipovaccine, and this appears after 2½ months. It is therefore possible that sufficient time was not allowed.

*The Production of the Typhoid-Carrier State.*—Gay and Claypole<sup>16</sup> have shown that the typhoid carrier state can be produced with regularity in rabbits and offers a means of testing the efficacy of any given method of prophylactic vaccination against typhoid.

In our work, each series of rabbits were tested against this carrier condition by the method of Gay and Claypole.<sup>8</sup> The culture used was *B. typhosus* 3 grown for many generations on 10 % rabbit blood agar. For each inoculation, a 24-hour culture was prepared on 10 % blood agar, suspended in sterile 0.85 % saline solution and 1 c c or 1/3 of the culture given intravenously. We attempted to standardize the dose

<sup>14</sup> Bull. de l'Inst. Pasteur, 1918, 16, p. 265; Compt. rend Soc. de biol., 1917, 80, p. 782.

<sup>15</sup> Jour. Am. Med. Assn., 1915, 71, p. 2146.

<sup>16</sup> Arch. Int. Med., 1914, 14, p. 671. Gay, F. P.: Typhoid Fever, 1918; also Footnote 8.

by using a uniform size of test tube, given the amount of culture slanted at a uniform angle and inoculated over the entire surface. In some animals,  $\frac{1}{6}$  of a culture was used and no difference noted.

Infection with *B. typhosus* produced in the vaccinated and unvaccinated control rabbits the characteristic syndrome. Of 10 normal rabbits, 8 or 80 % became carriers as against 94 % of Gay and Claypole. Of the 22 vaccinated rabbits, 3 of the 10 rabbits receiving the saline vaccine became carriers, or 30 %, and 4 of the 12 rabbits receiving lipid vaccine, or 33 $\frac{1}{3}$  %. The normal rabbits at times died acutely within 48 hours with marked loss of weight and malaise, but the majority showed a temporary loss in weight and became chronic carriers. In every case, the organism was recovered from the bile or blood and identified by agglutination with typhoid serum. All animals which survived were observed from 4-6 weeks and the agglutinin and antibody production studied, after which they were killed and necropsy examinations made. The only characteristic lesion in those which were proved to be chronic carriers was found in the gallbladder which usually was distended and enlarged and contained a large amount of light green, mucoid flocculated bile. Those successfully immunized gave negative cultures from the bile and presented a normal appearance. In the last series, the carrier condition was proved by removal of bile during life and the results verified by culture when the animals were killed later. In some cases, a second infecting dose of *B. typhosus* was given, but the carrier condition was still withstood.

#### DISCUSSION

Typhoid lipovaccine has not shown any antigenic properties in rabbits although the degree of protection afforded against the carrier state is practically equal to that of saline vaccine. This is an interesting point since it strongly supports the idea of Gay<sup>16</sup> that antibodies, particularly agglutinins, are an indication of the body reaction to the bacteria rather than a measure of the degree of protection afforded. This statement is supported by the experiments of Gay and Claypole<sup>16</sup> on sensitized and unsensitized typhoid vaccines and presents a condition analogous to that of lipid and saline vaccines. Gay and Claypole found that the agglutinin test was negative or weak after complete protection with sensitized vaccine, as compared with agglutinins formed by corresponding doses of unsensitized vaccine. Rabbits treated with sensitized vaccines were, however, more efficiently protected than the

ones treated with unsensitized vaccines. If we consider the mean agglutinin titer, before and after infection, in the entire series of rabbits, from the point of view of those that did or did not become carriers, we note that the saline vaccinated carrier rabbits show a titer of 1:1,280 as compared with the titer 1:320 in the noncarriers. This still further corroborates the discrepancy between antibodies and protection. Following inoculation, the titer rises to 1:1,280 in those becoming carriers and to 1:2,560 in noncarriers. The agglutinins in the lipid vaccinated animals are nil before inoculation in both the infected and noninfected, but reach the same agglutinin titer subsequent to inoculation.

This lack of agglutinin production by lipovaccine is at variance with the results obtained by a number of investigators. Whitmore and Fennel<sup>8</sup> did not state the agglutinin titer produced in typhoid, merely stating that it was present and assumed a degree of protection equal to that with aqueous vaccine. They found that dysentery lipovaccine produced, in rabbits, agglutinins of a titer of 1:3,200 for Shiga, 1:1,600 for Flexner and 1:2,400 for Bacillus "Y."

TABLE 2  
AGGLUTININ TITERS OF CARRIERS AND NONCARRIERS BEFORE AND AFTER INFECTION

	Mean Agglutinin Titer	
	Before Infection	After Infection
A. Carriers (15 rabbits)		
Saline vaccine .....	1:1280	1:1280
Oil vaccine .....	0	1:1280
Normal .....	0	1:1280
B. Noncarriers (17 rabbits)		
Saline vaccine .....	1:320	1:2560
Oil vaccine .....	0	1:1280
Normal .....	0	1:2560

Lewis and Dodge<sup>10</sup> stated that a typhoid lipovaccine, unheated, produces an agglutinin titer of 1:40 and in a few cases 1:100 and 1:500 and a heated vaccine 1:20 in a few instances only. A saline vaccine served as their control and produced agglutination 1:500. They therefore, concluded that a typhoid lipovaccine in a single dose is less efficient than a saline vaccine in three doses.

Tribondeau,<sup>14</sup> using a triple lipovaccine (T.A.B.) of Le Moignic-Pinoy, found that the agglutinins appear in man after the sixth day for all three organisms and continue for one month following vaccination. The largest number of serums agglutinated *B. typhosus* 1:1,000 and A and B, 1:500. The agglutinin production was constant from the 11th to the 25th day and was on a decline at about the 37th day. *B. typhosus* maintained a more constant level and did not decrease in



titer until the end of the second month. Observations were made, in our work, on the constancy of agglutinin formation, and we were unable to detect any evidence of decline over a period of six weeks following inoculation.

In the light of our data, we are of the opinion that specific antibodies are not demonstrable in oil vaccinated animals, but that their absence does not affect the protection afforded by such a vaccine. Blake and Cecil,<sup>17</sup> working on experimental pneumonia, used a pneumococcus lipovaccine prepared according to Whitmore and Fennel and failed to stimulate agglutinins or protective substances in the blood of monkeys. Saline vaccines did not produce agglutinins, but other protective bodies were present. However, they did find that lipovaccination influenced favorably the course of the disease so that the blood was not as heavily infected or even remained practically sterile. A single inoculation of saline vaccine failed to protect but modified the course of the disease and gave more encouraging results than one dose of lipovaccine or three doses of saline vaccine. Blake and Cecil concluded that saline vaccine is more likely to stimulate the formation of protective bodies in the blood and therefore gives a better degree of immunity. Saline vaccines stimulate agglutinin production, but their presence does not influence the degree of protection afforded to the animal. Lipovaccines do not show antigenic properties and afford a protection practically equal to that in the saline vaccinated.

#### CONCLUSIONS

The antigenic properties of typhoid lipovaccine in rabbits are not equal to those of saline vaccine. No agglutinins or fixation antibodies appear in the serums of those vaccinated with lipovaccine, while in those vaccinated with saline vaccine the mean agglutinin titer is 1:160.

Animals vaccinated with lipovaccine, whose serums show no agglutinin content, are nearly as well protected against becoming carriers as those vaccinated with saline vaccine whose serums show high agglutinin content. Even in the latter animals, the agglutinin content varies in degree inversely with the protection afforded. Therefore, the agglutinin titer is certainly not a measure of protection.

<sup>17</sup> Jour. Exper. Med., 1920, 31, p. 519.

## A METHOD FOR THE DETECTION OF PHENOLS PRODUCED BY BACTERIA

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The determination of phenols produced by the metabolic activities of organisms deserves consideration, since an accurate and sensitive method would furnish an additional means of distinguishing groups of micro-organisms whose cultural and morphologic characteristics are similar.

The method ordinarily used for the detection of phenol is essentially as stated by Eyre.<sup>1</sup> The culture is prepared in a flask containing nutrient broth and incubated. After incubation, 5 c c of 25% sulphuric acid is added to the cultivation, and the flask is connected with a condenser; 15 to 20 c c are distilled over, and the distillate divided into 3 portions, (a), (b) and (c). To (a), 0.5 c c of Millon's reagent is added and the solution is boiled; red color = phenol. To (b), 0.5 c c ferric chlorid solution is added; violet color = phenol. (If the distillate be acid the reaction will be negative.) To (c), bromine water is added; crystalline white precipitate of tribromphenol = phenol. In case indol and phenol appear to be present in cultivations of the same organism, it is recommended that they be separated before testing. This is done by redistilling the first distillate (which would contain both indol and phenol) after rendering it alkaline with caustic potash. The distillate contains indol, the residue phenol. The residue is saturated with CO<sub>2</sub> and redistilled. The last distillate will contain phenol and is tested as stated in the foregoing.

Rettger,<sup>2</sup> in his study of the chemical products of *B. coli* and *B. lactis-aerogenes*, used a somewhat similar method. The medium used in his experiments was an egg-meat mixture, and incubation was carried on in the presence of hydrogen. Portions of 400 c c of the decomposition mixture were diluted with an equal volume of water, and after

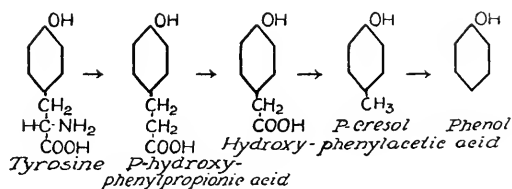
Received for publication June 7, 1921.

<sup>1</sup> Bacteriological Technique, 1916, p. 287.

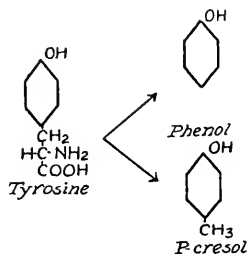
<sup>2</sup> Am. Jour. of Physiol., 1903, 8, p. 284.

the addition of 5 c c of dilute sulphuric acid, were distilled with steam until from 600 to 700 c c of distillate had formed. The distillate was made alkaline with KOH, and again distilled with steam until 500 c c of liquid had collected. Indol and skatol were in the distillate. The last distillation residue was saturated with CO<sub>2</sub> and distilled until 500 c c of liquid had collected. Phenols were in this distillate.

The mother substance of phenol is tyrosine, possibly phenylalanine or other aromatic amino acids, if any exist, other than tryptophane. The exact manner in which tyrosine is decomposed in putrefaction is not certainly known. The transformation, as suggested by Matthews,<sup>3</sup> may be as follows:



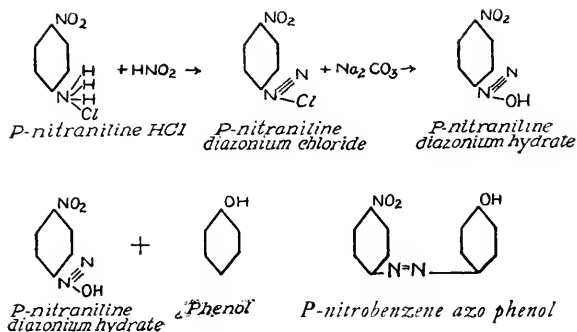
It is quite possible that tyrosine is split directly to yield phenol or p-cresol:



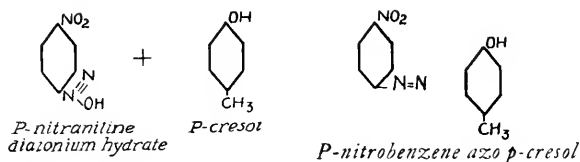
The method proposed in this paper is dependent on the formation of an azo-dyestuff, brought about by the reaction of a diazotized aromatic amine and a phenol in alkaline solution. The formation of such a colored compound affords an extremely sensitive method for the detection of minute quantities of phenol. In considering a suitable amine for coupling with the phenol, paranitraniline was chosen, since diazotized paranitraniline will couple practically quantitatively and

<sup>3</sup> Physiological Chemistry, 1916, p. 745.

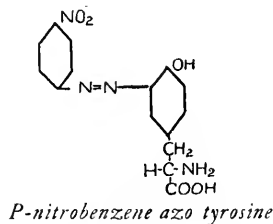
instantaneously, especially so in dilute solutions. The reaction is as follows:



With P-cresol, we would have:



An important advantage of this test is that indol will not couple with diazotized paranitraniline. It is important to note that tyrosine is capable of uniting with a diazotized aromatic amine to form azo dyestuffs. With paranitraniline, the resulting dyestuff would have the following constitution:



Phenylalanine, on the contrary, would not be capable of such union, since it is neither an aromatic amine nor a phenol.

From these facts, it is evident that phenol or p-cresol could be detected in a bacterial culture by resorting to distillation of the acidified culture with steam, and after alkalinizing the distillate, adding diazo-

tized paranitraniline and noting the formation of the azo dye. As stated the presence of indol in the distillate would not interfere.

The procedure recommended in applying the test is as follows:

1. Cultivation: The organism to be tested is grown, most conveniently in a 1 liter flask, on a suitable medium, such as nutrient bouillon, egg-meat mixture, egg-bouillon, etc., incubation being carried on under aerobic, anaerobic or partial tension conditions. After incubation, the culture is ready for distillation.

2. Distillation: The flask containing the culture is connected up with a steam distillation apparatus. In order to avoid any mechanical carrying-over of medium, the apparatus was set up as shown in fig. 1.

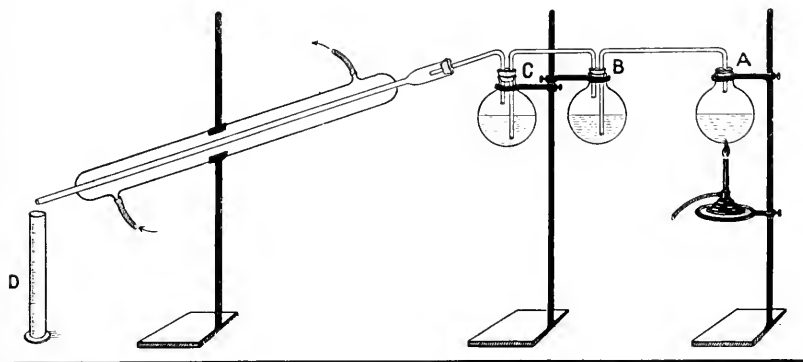


Fig. 1.—The flask was used to furnish steam for the distillation. The steam evolved caused the medium in B, which had been acidified previously with sulfuric acid, to boil, thus producing steam which in turn carried phenols over into C. C contained distilled water, acidified with sulfuric acid. The steam evolved from B caused the water in C to boil, the distillate containing phenols being collected at D. The distillate was made distinctly alkaline by the addition of 10% sodium carbonate.

3. Preparation of Diazo Solution: One-tenth gm. of pure paranitraniline is placed in a test tube and 0.6 c c concentrated HCl added, heat being applied to hasten solution. The solution is then diluted to 5.0 c c with distilled water and 1.0 c c of M/1  $\text{NaNO}_2$  added. The solution is allowed to stand for 5 minutes; then it is added drop by drop to the distillate, care being taken that the distillate is kept alkaline. The appearance of a red color indicates phenols. In extreme dilutions the color appears yellowish-red. The observer must not be deceived by the formation of a light yellow color, as this is due to paranitraniline diazonium hydrate.

This method will show on blank determinations, a distinct yellowish-red color with phenol diluted 1 part to 1,000,000. In culture mediums,

it is easily possible to show the presence of phenol in concentrations of 1:500,000. The sensitiveness of this reaction is especially valuable, when we consider that the reagents heretofore used will not show their characteristic colorations in dilutions as low as 1:10,000. Furthermore, the presence of other compounds, such as indol, interferes with ferric chloride and Millon's reagent, necessitating additional work in separating the two before testing. As tyrosine and derivatives of tyrosine, in addition to phenol and p-cresol, will couple with the formation of an azo dye similar to those formed by phenol and p-cresol, it is important to state that tyrosine and, so far as known, split-products of tyrosine, other than phenol and p-cresol, will not distill over with steam.

In the near future, a quantitative method based on the above will be given, also experiments involving phenol production by various species of bacteria.

# EXPERIMENTAL MEASLES IN RABBITS AND MONKEYS\*

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In our search of the literature on experimental measles we have been unable to find any mention of attempts having been made to transmit the virus to rabbits. Anderson and Goldberger<sup>1</sup> speak of the apparent insusceptibility of animals other than the monkey to the inoculation of measles virus, but no reference is given.

In order to test the receptivity of rabbits to the infection of measles, the blood of 6 patients with measles was inoculated into 6 rabbits. Three of the rabbits were inoculated with blood drawn on the second day, 2 rabbits with blood drawn on the third and 1 with blood drawn on the fourth day after the onset of the disease. In all we inoculated 17 rabbits, 6 with human blood and 11 with the blood of rabbits that had given evidence of a reaction. The inoculations were given intravenously in amounts varying from 1 to 15 cc. All of the rabbits inoculated with human blood gave evidence of a reaction. So also did all but 2 of the rabbits subinoculated from these 6 rabbits. Passage inoculations for subsequent injections into monkeys were carried out only from patient "S."

## SYMPTOMS IN RABBITS

Fifteen rabbits developed symptoms in from 3 to 7 days. The symptoms were not so marked as in monkeys. In some instances there was a rise in temperature coincident with a decrease in the total leukocyte count, but this was by no means constant. We noted also that in the majority of the rabbits there was frequently a leukocytosis on the second, third or fourth day after injection followed by a decrease in the total leukocyte count. The fact that these findings were not constant, together with the fact that we had found great fluctuations in the daily counts of these animals for one week prior to their inoculation, led us to disregard these changes in the white blood counts.

Received for publication June 6, 1921.

\* This work is part of an investigation on respiratory infections. The investigation was supported in part by a grant from the Influenza Commission of the Metropolitan Life Insurance Company.

<sup>1</sup> U. S. Public Health Reports, 1911, 26, p. 847.

Of the 15 rabbits showing symptoms of measles, 10 developed small hyperemic, slightly elevated spots on the labial mucosa; in 5 instances these spots showed whitish centers. Twelve rabbits developed in from 2 to 4 days, a marked conjunctivitis with a moderate edema of the lids and lacrimation. Four rabbits developed a slight diarrhea.

In from 3 to 8 days all of the 15 animals showed erythema of varying degrees, from a slight flush over the chest and in the axillary region to a general redness extending to the groin. A redness of the skin always followed the shaving of the rabbits, but, unlike the control animals noted in the following, the flush became more intense in from 3 to 8 days.

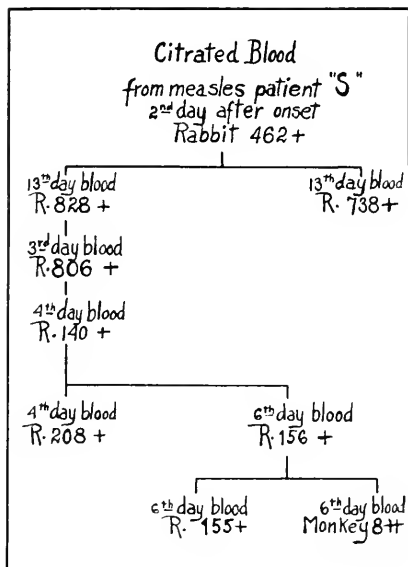
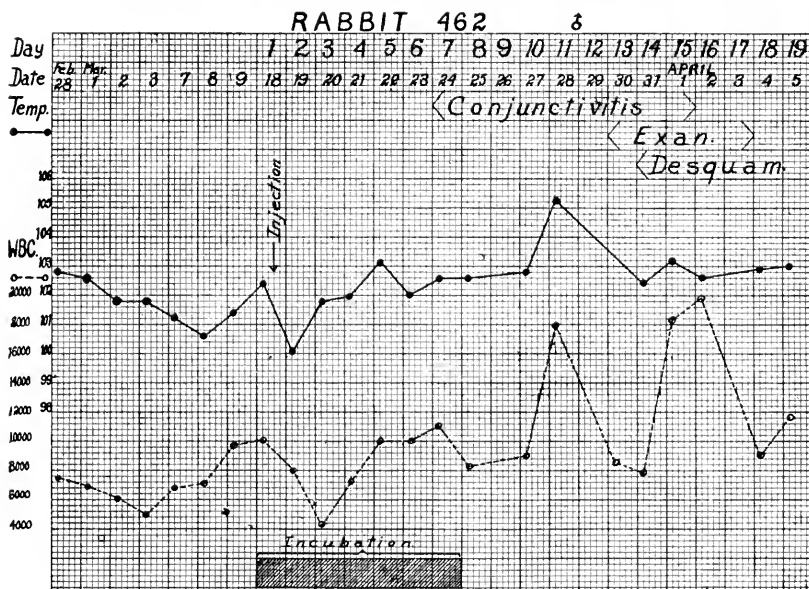


Chart 1.—Transmission of measles virus from case "S" through 5 rabbits to a monkey; suggestive reaction +, typical measles reaction ++.

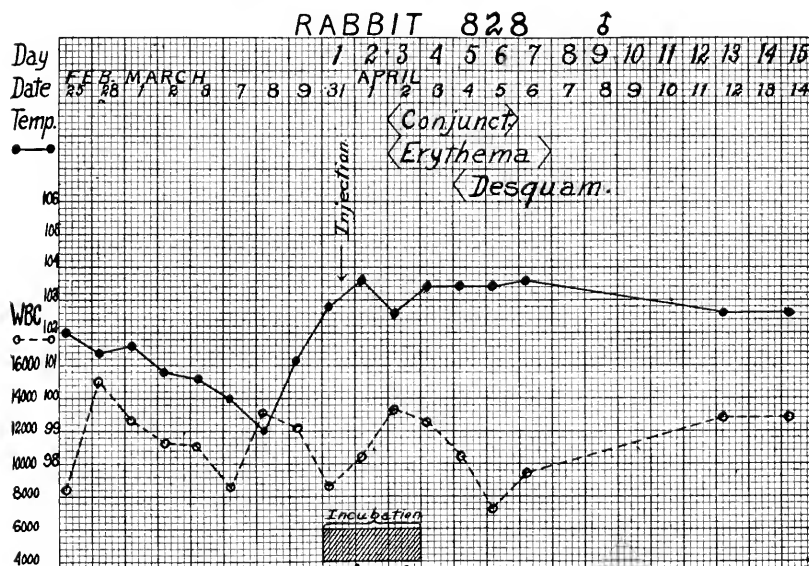
In no instance was a distinctly typical exanthem noted. In 8 rabbits we noted several erythematous macules on the chest and abdomen. Such a rash was not distinctive. Neither is the reaction on the skin of rabbits after inoculation with cowpox vaccine the typical one that takes place in human beings.

In all of the 15 rabbits a marked desquamation occurred, beginning from the fifth to the fourteenth day. Both the shaved and unshaved portions of the neck, chest, axillae, abdomen and groin were involved.





A



B

Chart 2.—Transmission of measles virus, strain from case "S," by means of intravenous inoculation of whole citrated blood; A, rabbit 462 from case "S"; B, rabbit 828 from rabbit 462.

In two instances the desquamation was fine and branny; with the remaining 13 animals large flake-like scaling took place.

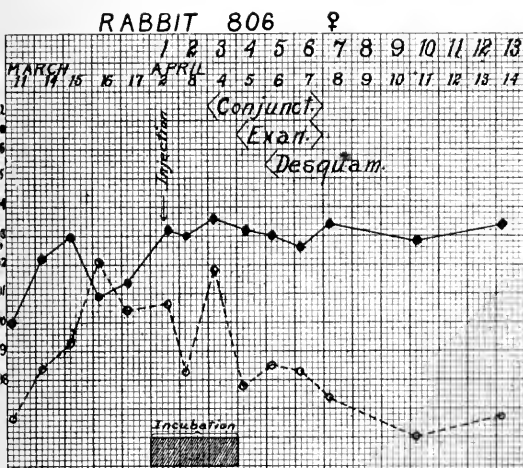
From one of the human cases, "S," the virus was passed through 5 rabbits (Charts 1, 2 and 3). On the sixth day after inoculation, the fifth rabbit was bled from the heart and the citrated blood injected intravenously into monkey 8, *M. rhesus*. The monkey developed a leukopenia in 3 days (Chart 4A). On the fourth day there was loss of appetite, slight reddening of the eyelids and lacrimation with a marked erythema over the face, neck and shoulders, while on the labial mucosa two discrete, elevated, hyperemic macules with bluish white centers appeared. On the fifth day a maculopapular rash appeared about the mouth, cheeks and forehead. On the sixth day, over the bluish abdomen of the monkey, small clusters of yellowish, elevated papules appeared. From the sixth to the ninth day there was a moderate erythematous, granular rash on the mucous membrane of the lips. The papules on the labial mucosa disappeared on the eighth day. The rash also began to fade and marked desquamation was noted on the face, chest, shoulders, abdomen and in the groin.

Blake and Trask<sup>2</sup> have shown that monkeys inoculated intratracheally with nasopharyngeal washings from patients with measles develop characteristic symptoms of measles. Accordingly, we reinoculated monkey 8, *M. rhesus*, one month later with nasopharyngeal washings containing another strain of measles virus (case "O"). The monkey failed to react (Chart 4B). The control (monkey 24, *M. rhesus*, chart 5A) developed the characteristic symptoms of measles in 6 days.

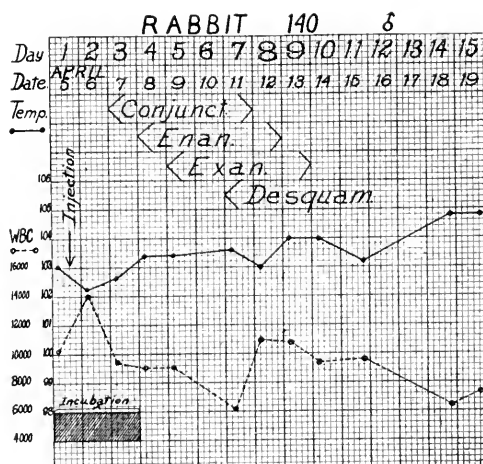
In order to test the receptivity of the monkey to the infection of measles with blood from a human being with measles the following experiment was made:

Two patients with measles, "Y" and "O," were bled on the third day after the onset of the disease. In both cases Koplik spots were present. Conjunctivitis and coryza were present in one case only. Both cases showed a maculopapular rash. The citrated blood of these two patients was pooled and 10 cc were inoculated intravenously within an hour into monkey 26, *M. rhesus* (chart 5B). On the fourth day after inoculation the animal was drowsy; loss of appetite was noted, and the diminution in the total leukocyte count was marked. The monkey developed no distinct symptoms until the sixth day when a few bright red spots, slightly elevated, were noted on the labial mucosa. On the seventh day discrete, slightly raised, hyperemic macules with

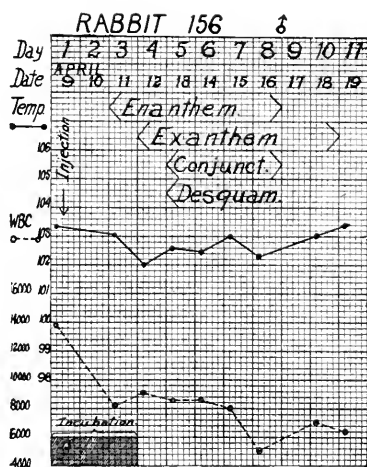
<sup>2</sup> Jour. Exper. Med., 1921, 33, p. 385.



A



B



C

Chart 3.—Transmission of measles virus strain from case "S" by means of intravenous inoculation of whole citrated blood; A, rabbit 806 from rabbit 828; B, rabbit 140 from rabbit 806; C, rabbit 156 from rabbit 140.

bluish white centers were present on the inside of the cheeks; the enanthem on the labial mucosa was more marked but not coalescent. On the eighth day we noted a few flat reddish macules, disappearing under pressure, on the face and shoulders. Conjunctivitis appeared on the ninth day; the lids were thickened and hyperemia and lacrimation were present. The following day the exanthem had spread to the chest and abdomen, while the rash on the face of the monkey showed a yellowish brown pigmentation. During the next 4 days the exanthem gradually faded, the spots on the chest and abdomen becoming a yellowish brown also. Desquamation started on the eighth day, but was never marked. By the fourteenth day the animal appeared well; the

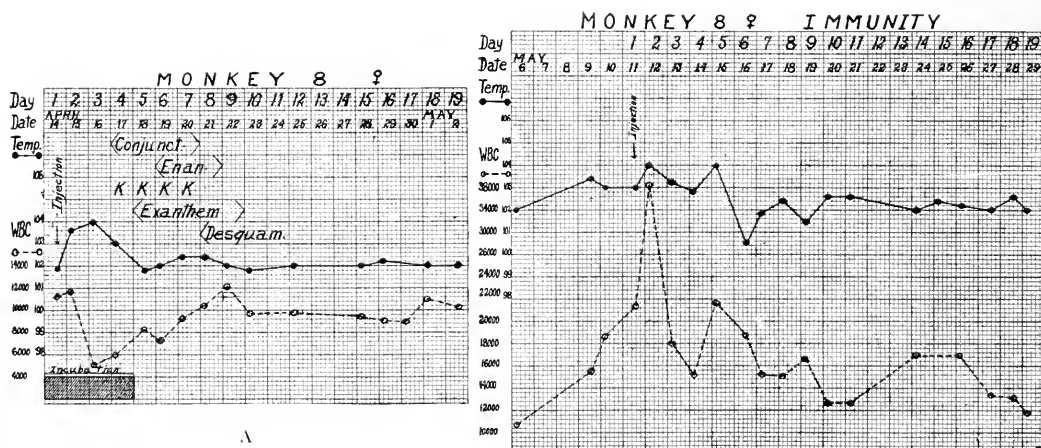


Chart 4.—Observations on monkey 8; A, transmission of measles virus strain from case "S" by means of intravenous inoculation of whole citrated blood from rabbit 156 (chart 3C); B, attempted reinfection by means of intratracheal inoculation with 10 c.c. of nasopharyngeal washings containing another strain of measles virus (case "O"). The rise in the total leukocyte count prior to inoculation was due, in all probability, to a slight diarrhea. This condition rapidly ceased on change of food. As Blake and Trask have noted, the sharp rise following the injection of nasopharyngeal washings is probably due to the effect of other organisms. This animal showed no reaction to the virus and was discharged on the nineteenth day. Control monkey 24 (chart 5A), inoculated with 10 c.c. of the same washings developed the characteristic symptoms of measles in 6 days.

exanthem had entirely faded and only a slight pigmentation and desquamation were noted.

**Controls.**—Eleven rabbits were shaved over the neck, chest, axillae, abdomen and groin. Six rabbits were inoculated intravenously each with 5 cc of citrated blood as follows: two with blood from two cases of diphtheria, one from a case of lobar pneumonia, one from a case of lethargic encephalitis, one with normal human blood and one with normal

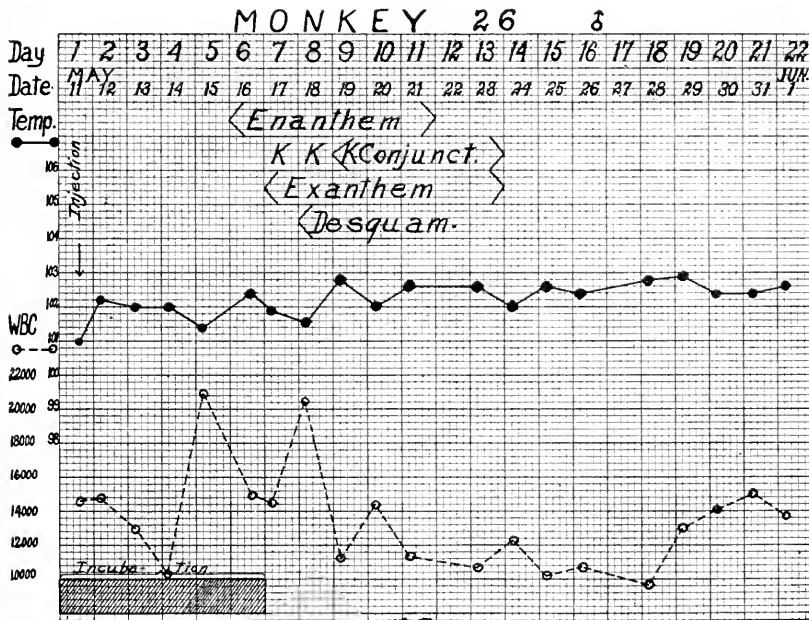
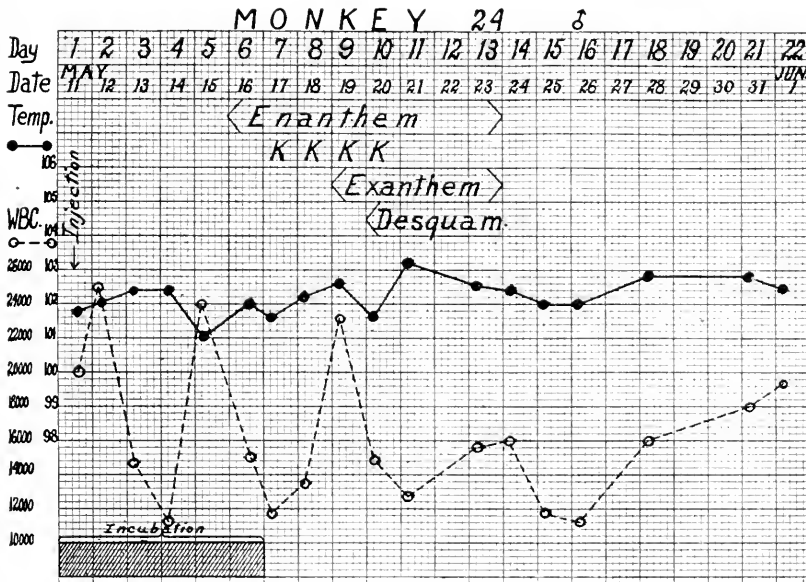


Chart 5.—A, observations on monkey 24 inoculated intratracheally with 10 c.c. of nasopharyngeal washings from case "O"; B, observations on monkey 26 inoculated with 10 c.c. of whole citrated pooled blood from measles patients "Y" and "O".

rabbit blood. The total leukocyte count of all these rabbits showed considerable variation from day to day with the exception of the rabbit inoculated with normal rabbit blood. This count remained fairly constant.

The 5 uninoculated rabbits were kept under observation for the appearance of an erythema and desquamation. All of the 11 control animals showed a redness of the skin incident to shaving. This flush disappeared in from 48 to 72 hours, whereas in the animals showing evidence of a reaction to the inoculation of blood from patients with measles, the redness became more marked from the third to the eighth day following the inoculation and persisted for from 3 to 5 days and then gradually faded. In no instance was desquamation noted in the 11 control rabbits.

Microscopic examination of scrapings made from the spots on the labial mucosa showed no parasitic invasion.

Aerobic and anaerobic cultures made of blood prior to inoculation showed no evidence of growth.

#### SUMMARY

Blood from 6 patients with measles was inoculated into rabbits. The animals gave evidence of infection.

Passage from one human case of measles was carried on through 5 rabbits, and a monkey inoculated with the blood of the fifth rabbit gave typical symptoms of measles.

A monkey inoculated with pooled blood from two human cases of measles taken on the third day after the onset of the disease gave the characteristic symptoms of measles.

Blood from cases other than measles when inoculated into rabbits failed to give evidence of infection.

# THE RELATIONSHIP OF THE PNEUMOCOCCUS TO ACUTE INFECTIONS OF THE UPPER RESPIRATORY TRACT IN MAN\*

## INFLUENZA STUDIES VI

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In the present study, two general groups of acute respiratory infection have been investigated. The first group included a number of simple acute respiratory infections of varied clinical type, occurring in an urban population, and broadly classified as "common colds." The second was a recurrent epidemic of influenza occurring in the early winter months of 1920 and characterized by the same general clinical picture which was manifested in the great wave of influenza pandemic in 1918. The purpose of the work was to determine, first, the frequency with which the pneumococcus could be demonstrated in cases of influenza and in waves of common colds, and then to compare the extent of its incidence in these conditions with its occurrence in the upper respiratory tract of normal persons.

In the event that the pneumococcus was present to a greater extent in the pathologic respiratory tract than in the normal, it was essential to determine whether those pneumococci represented a single like strain in a given group of cases, or whether they were heterologous in nature and representative of unrelated varieties of that organism. Likewise, the pneumococcus isolated from the site of the primary lesion in common colds and influenza possesses a number of possibilities in its relationship to the infection. It may be present there normally as a part of the normal bacterial flora of that region and be involved in no way with the infection. It may, although normally present, be playing an accessory rôle in the inflammation. Again, it may be present as a secondary invader to the bacterium or virus causing the original infection. Lastly, it may be the true etiologic agent involved in the inflammation.

Received for publication June 3, 1921.

\* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York. Part of the expense of these studies has been met by a grant from the University of Chicago.

With these various possibilities in mind, it was hoped by the general procedure outlined to acquire a certain amount of information bearing on the rôle of the pneumococcus in the etiology of "common colds" and influenza.

#### BIOLOGIC DIFFERENCES AMONG PNEUMOCOCCI

The study of the pneumococci isolated from patients with common colds and influenza and from normal persons, and the search for a particular organism which might be habitually concerned in the etiology of a given infection, has been largely facilitated by the advance, within the last few years, of our knowledge of the biology of the pneumococcus. It has been demonstrated that there exists within the species, certain well differentiated groups or types distinct one from the other.

Eyre and Washbourn,<sup>1</sup> in testing a serum made by Pane,<sup>2</sup> an Italian worker, for antibodies which would protect white mice against lethal doses of certain stock strains of pneumococci, found that in only 2 of 5 cases would this serum protect against his strains of cocci. Protection was not afforded against the other 3 in any appreciable dilution. This was the first indication of a biologic difference in strains of pneumococci.

Some years later Park and Williams<sup>3</sup> showed that the organism described originally by Schottmüller<sup>4</sup> as *Streptococcus mucosus* was really a pneumococcus, judged by its biologic properties, and should be classified as such, although differing morphologically from other varieties of pneumococci. Added evidence tending to include these organisms among the pneumococci was furnished by Collins<sup>5</sup> through specific agglutination and agglutinin absorption tests. She furthermore contributed data tending to show that pneumococci in general could be separated into various groups by means of their agglutinative properties. Added observations by Hanes<sup>6</sup> show decisively that these organisms are pneumococci, and that there is distinct cross agglutination among various members of the group. Neufeld and Händel<sup>7</sup> demonstrated that there were well established varieties of pneumococci which, though resembling each other morphologically, nevertheless showed distinct immunologic differences. The work of Neufeld and Händel was followed by a more detailed study by Cole<sup>8</sup> and by Dochez and Gillespie,<sup>9</sup> the latter workers dividing pneumococci into 4 types or groups. These groups they designated as types 1, 2, 3 and 4. Types 1 and 2 differed from each other only slightly in morphology, but immunologically they were entirely distinct. These immunologic differences they demonstrated by animal protection and by agglutination tests. Group 3 included those organisms designated as *Pneumococcus mucosus* and were distinct from other pneumococci morphologically and serologically, although agreeing among themselves as a group. Group 4 was a heterologous division and included all cultures which did not fall into one of the other 3 groups. They resemble the organisms of types 1 and 2 morphologically, but do not possess common agglu-

<sup>1</sup> Brit. Med. Jour., 1899, 2, p. 1247.

<sup>2</sup> Centralbl. f. Bakteriöl., 1897, 21, p. 664.

<sup>3</sup> Jour. Exper. Med., 1905, 7, p. 403.

<sup>4</sup> München. med. Wehnschr., 1903, 50, p. 849.

<sup>5</sup> Jour. Exper. Med., 1905, 7, p. 420.

<sup>6</sup> Ibid., 1914, 19, p. 38.

<sup>7</sup> Arb. a.d.k. Gesndhtsamte, 1910, 34, p. 293.

<sup>8</sup> Arch. Int. Med., 1914, 14, p. 56.

<sup>9</sup> Jour. Am. Med. Assn., 1913, 61, 727.



tinative properties either with the other groups or among themselves. This classification has been substantiated by subsequent investigations, those of Lister,<sup>10</sup> Mathers,<sup>11</sup> Clough,<sup>12</sup> Hartman and Lacy,<sup>13</sup> Richardson,<sup>14</sup> and Armstrong<sup>15</sup> among others.

Later work by Avery<sup>16</sup> on the classification of the group, showed that there were certain strains of pneumococci which reacted atypically when agglutinated with type 2 serum in that there occurred a partial or delayed reaction. Closer study led to a separation of these atypical type 2 strains into 2 well-defined serologic subgroups, type 2a and 2b with a heterologous subgroup type 2x. Avery's experiments were extended by Stillman,<sup>17</sup> and study of a larger number of such strains proved that atypical type 2 organisms could be subdivided into at least 12 different subgroups. In fact, it would seem that there is really evidenced within this group a repetition of what has occurred in the whole series. Type 2 has a tendency to variation inherited from the original coccus, with one of the subgroups type 2m still showing tendencies to subdivide.

Attempts to show some agreement among the heterologous type 4 strains failed to demonstrate distinct division into subgroups. Fourteen strains studied by Dochez and Gillespie,<sup>9</sup> seem generally to represent distinct varieties (Cole<sup>8</sup>). Olmstead,<sup>18</sup> however, reports, from a study of 213 group 4 strains, that they can be divided into about 12 subgroups by cross agglutination experiments.

#### METHODS OF STUDY

The method of collecting material for subsequent examinations is of importance, and preliminary experiments were made to determine the most favorable procedure. The collection of sputum was out of the question because bronchial secretions are not raised in the majority of cases. All material was obtained therefore, by swabbing the mucous membrane with sterile cotton swabs placed on iron wire, after the type of the Mathers swab.<sup>19</sup>

All patients were examined clinically by another member of the staff, Dr. W. B. Sharp, who was working at the same time on the epidemiologic and clinical phases of acute upper respiratory infections. I am indebted to Dr. Sharp for all clinical data contained in the present report. Depending on the clinical diagnosis, certain areas of mucous membrane were thoroughly stroked with the swabs.

The first 25 cases included 13 of acute rhinitis, 2 of acute follicular tonsillitis, 5 of acute pharyngitis, and 5 cases which presented involve-

<sup>10</sup> South African Institute for Medical Research (Publications), Dec. 22, 1913.

<sup>11</sup> *Jour. Infect. Dis.*, 1915, 17, p. 514.

<sup>12</sup> *Bull. Johns Hopkins Hosp.*, 1917, 28, p. 306.

<sup>13</sup> *Jour. Am. Med. Assn.*, 1917, 69, p. 2165.

<sup>14</sup> *Jour. Lab. & Clin. Med.*, 1919, 4, p. 484.

<sup>15</sup> *Brit. Med. Jour.*, 1921, p. 259.

<sup>16</sup> *Jour. Exper. Med.*, 1915, 22, p. 804.

<sup>17</sup> *Ibid.*, 1919, 29, p. 251.

<sup>18</sup> *Proc. Soc. Exp. Biol. and Med.*, 1916, 14, p. 29; *Jour. Immunology*, 1917, 2, p. 425.

<sup>19</sup> *Jour. Infect. Dis.*, 1918, 22, p. 523.

ment of the bronchi together with acute inflammation of the upper respiratory tract. In order to obtain data on the most favorable site from which to obtain material, separate swabs were taken from the nose, the nasopharynx and the pharynx. Individual examinations were made for the pneumococcus from each of the swabs so taken, according to the methods outlined later. Briefly, it was found that cultures from the nasopharynx gave the greatest percentage of pneumococci irrespective of diagnosis and indicated that region to be a primary seat of localization for the organism in acute respiratory infections of this class. In cases of rhinitis, throat cultures were found to be of little value, but the nasal cultures on two occasions gave positive results when material was taken well back toward the choanae, while nasopharynx cultures proved negative. In inflammations of the pharynx, larynx and tonsils, nasal cultures proved of no value, indeed cultures from the region of the pharynx were positive for the pneumococcus in only 1 of 6 cases. As a result of these preliminary examinations the following routine procedure was adopted: In cases of rhinitis, the middle fossa and floor of the nostril was swabbed, together with a second swabbing of the nasopharynx. In cases of tonsillitis, pharyngitis, laryngitis and influenza, swabbings were made from the posterior pharynx and from the nasopharynx. Normal persons were swabbed in all three areas, nasopharynx, nostril, and the pharynx. Particular care was taken in all swabbings to prevent salivary contamination. Separate determinations were not made. The two swabs were pooled, and a single determination made from the combined material by injection into mice.

The methods for the demonstration of pneumococcus in the material collected as described offered certain technical difficulties. Early in the studies, the swab was placed in a tube of sterile, warmed Ringer solution, and the mucous secretions brought into suspension by allowing the swab to stand in the warm fluid for about 2 hours with frequent agitation. One c c of the suspension was then injected intraperitoneally into a white mouse. The method first advanced by Blake<sup>20</sup> for the determination of the presence and type of pneumococcus in sputum was then followed, essentially as recommended by him. The results were not satisfactory in that few pneumococci were obtained, and the mortality of the mice was very low. Two swabs were taken from a given area and treated in the same manner as had been the single swab;

<sup>20</sup> Jour. Exper. Med., 1917, 26, p. 67.

it was hoped thereby to obtain a heavier inoculum. No appreciable improvement could be observed. Finally, the method was modified to include a preliminary enrichment of the material on the swabs, followed by subsequent inoculation of this enriched fluid into mice. This modified method has given excellent results and is essentially as follows:

The two swabs taken from the most promising regions, as determined by clinical examination, the nasopharynx and either the pharynx or nasal cavity, were placed in 5% sheep dextrose blood broth, made according to the method which Avery<sup>21</sup> used in his substitute method for mouse technic in the typing of pneumococci from sputum. Our technic differed only that sheep blood was substituted for rabbit blood. The broth was then incubated for a period of from 8 to 12 hours at 37 C., with agitation of the tube from time to time to insure proper distribution and full benefit from the added blood. This method gives a preliminary enrichment of the pneumococci contained on the original swabs. Stained smears from such broth cultures will ordinarily show a preponderance of pneumococci over the accompanying staphylococci, within the time of incubation used. Following the preliminary enrichment, 1 cc of the broth culture, as free as possible from red blood corpuscles, was injected intraperitoneally into white mice. In cases in which death of the animal did not follow within 24 hours, a peritoneal puncture was made. Smears were made from the fluid withdrawn and, satisfactory growth being evidenced, the animal was killed.

The peritoneal cavity of the mouse was washed according to the method of Blake<sup>20</sup> and the pneumococci typed by agglutination and precipitin tests in the usual manner. Cultures on blood-agar plates were made at necropsy, from peritoneal fluid and from heart blood. The gram-positive, lance-shaped, encapsulated diplococci were later isolated in pure culture, and confirmatory agglutination and bile solubility tests were made with the pure broth culture for final determination of the type of pneumococcus. All agglutination tests were given 2 hours' incubation at 37 C., and a preliminary reading made. They were then placed in the icebox over night and final readings made in the morning. Bile solubility tests, as the final criterion of a pneumococcus, were made by adding one part of ox bile, first filtered and then sterilized by steaming for 3 succeeding days, to 3 parts of a

<sup>21</sup> Jour. Am. Med. Assn., 1918, 70, p. 17.

broth culture of the organism. In all instances final diagnosis of organism and type were based on these reactions in pure broth culture.

The experience of many workers has seemed to indicate that passage of suspected pneumococcus material through a white mouse furnishes probably the most exact technic for its isolation. The animal tissues furnish a favorable environment for the growth of the organism, and in addition the blood stream of the white mouse exercises a more or less specific filtering action on the pneumococcus. Nevertheless, it was felt that careful examination of plate cultures from the swabs might yield even better results. Consequently, in a series of 56 cases, duplicate plate examinations and mouse determinations were carried out. The medium used for the plates was a 5% sheep blood veal infusion agar, reaction PH 7.8.

The plates were inoculated with the swabs and then streaked out in sunburst fashion. After 24 hours' incubation at 37 C., they were examined under the dissecting microscope and 5 green-producing colonies, selected at random from different portions of the plate but showing minute differences in colony structure, were picked to blood agar slants. These were later obtained in pure broth culture and subjected to bile solubility tests. The mouse technic was as previously outlined.

Of the 56 cases subjected to duplicate examinations, 13 proved positive by the mouse method while only 4 gave positive cultures of pneumococci by the plate method. In only one instance was a pneumococcus found by the plate method and not obtained by the mouse technic. The results show that the technic of enrichment and subsequent mouse inoculation is best suited to the isolation of pneumococci present in these conditions. Following these preliminary experiments in the autumn of 1919, the mouse method was employed exclusively in further studies.

#### THE INCIDENCE OF THE PNEUMOCOCCUS IN NORMAL PERSONS

The incidence of the various types of pneumococci in the pneumonias has been determined for a large number of cases, not only by the original workers, Dochez and Gillespie, but by numerous other investigators previously cited; likewise, data are at hand bearing on the evidence of the organism in normal persons.

Pneumococci are frequently found in saliva. Early studies by Frost, Divine and Reineking<sup>22</sup> demonstrated pneumococci in the saliva of 18 of 50 different normal persons examined, or 36%, although their criteria for determination of the coccus were probably not exact in the light of our present-day knowledge. Study of the sputa from 70 normal persons by Park and Williams<sup>3</sup> demonstrated pneumococci in 51 instances, although the same criticism might apply to their work, in that bile solubility tests were not employed. Stillman,<sup>23</sup> in a comprehensive study conducted some years later, and covering about 297 cases of normal persons, demonstrated the organism in 39% of his cases. The incidence of types was also determined. He found that types 4 and 3 were the most common groups of pneumococcus observed in normal saliva, with atypical type 2 organisms showing a relatively high incidence. The fixed types, types 1 and 2, were found in only a single instance. It would seem that the incidence of the pneumococcus in saliva is relatively high, averaging probably 40%, with certain definite types predominating.

A distinct necessity in the study of an organism concerned in acute respiratory infections is the determination of a mean for that organism in normal persons in sites most subject to acute inflammation. Certain data are available concerning the incidence of the pneumococcus in the nasopharynx and pharynx of such normal persons. Sailer, Hall, Wilson and McCoy<sup>24</sup> studied 700 men in a military command. These men were free from respiratory infection, but had been in more or less contact with prevailing pneumonia. Swabs were taken from the nasopharynx, spread on blood-agar plates, typical colonies picked, and broth cultures subjected to the usual tests of bile solubility and agglutination for type. One hundred and eleven of these cases yielded cultures of the pneumococcus, or 16% of the total. Of the positive findings 5.4% were of type 1, 13.52% were type 2, type 3 constituted 4.5% while 76.58% were found to fall into the type 4 group. A later study of a civilian population by Meyer<sup>25</sup> showed 21 of a series of 100 normal subjects to be carriers of the pneumococcus, or 21%. Of the 21 cultures isolated, none were classified as members of the fixed types 1 and 2. One type 2a strain was identified, while 3 proved to be of type 3. The remaining 17 belonged to the heterologous type 4. Fifty samples

<sup>22</sup> Jour. Infect. Dis., 1905, Suppl. 1, p. 298.

<sup>23</sup> Jour. Exper. Med., 1917, 26, p. 513.

<sup>24</sup> Arch. Int. Med., 1919, 24, p. 600.

<sup>25</sup> Jour. Am. Med. Assn., 1920, 75, p. 1268.

of sputum from various persons among a dispensary population were studied by Sydenstricher and Sutton<sup>26</sup> and gave similar results, in that 38% were positive for the pneumococcus, with type 4 predominating.

A further study of pneumococcus incidence among normal persons by the same methods employed for the study of respiratory infections was felt to be essential, and preliminary to any studies of the nose and throat under pathologic conditions. Briefly, 46 cultures have been made from a group of students in the university who had not at the time been knowingly exposed to colds, or who had themselves suffered from colds within recent date. All were subjected to a careful clinical examination before cultures were taken to rule out possible inflammation of the respiratory mucous membrane. From the cultures derived from 10 of these persons, pneumococci were obtained, 21.7%. The majority of these were type 4, nine of the cultures falling into this group. The other culture was of atypical type 2 variety. Strains representative of the fixed types were not found, nor were strains of type 3 isolated. The swabbed material, even after preliminary enrichment, was in general nonpathogenic, inoculations of 1 c.c. of the blood broth culture from swabs, producing death of the mice within 24 hours in only 2 instances in the entire series. The incidence of the pneumococcus, then, in the normal respiratory tract was found to be somewhat over 21%, with most of the organisms type 4, and possessing a low grade of virulence.

#### THE PNEUMOCOCCUS IN COMMON COLDS

The results obtained by a considerable number of investigators previously cited, has led to definite knowledge of the incidence of the different types of pneumococci in pneumonia; likewise, the incidence in normal persons is rather well understood. Few studies, however, have been made on the prevalence of the types of pneumococci in simple acute respiratory conditions. Valentine<sup>27</sup> has probably made the most extensive study of the pneumococcus in common colds. Her investigations were directed toward a determination of the pneumococcus in these simple infections as a possible source of contagion for lobar pneumonia. Cases in her series numbered 65, and pneumococci were recovered from 43 of them. The types were represented by two strains of type 1, two of type 2, four of type 3, while 35 were placed in the heterologous type 4.

<sup>26</sup> Bull. Johns Hopkins Hosp., 1917, 28, p. 312.

<sup>27</sup> Jour. Exper. Med., 1918, 27, p. 27.

Among the pneumonia cases studied by Clough<sup>12</sup> were included a few cases of acute and chronic bronchitis in which pneumococci were found. Other than these two reports, no information bearing on the frequency of pneumococci in the simpler respiratory conditions could be found in the literature.

In studying the cases of common colds in our series, no attempt was made to select certain types of infection. All cases among a student population were examined as they were reported from time to time. Consequently, they vary considerably in the clinical type of infection. A special effort was made, however, to have cases available early in the onset of the inflammation, and the majority were observed within the first 24 hours of the cold, rarely after a period of more than 48 hours following the onset of symptoms. A total of 77 colds are included in the present series. Briefly, 27 of them showed the pneumococcus when cultivated by the methods outlined. The incidence of the different types of pneumococci is indicated in table 1.

TABLE 1  
THE PNEUMOCOCCUS IN COMMON COLDS, TOGETHER WITH THE INCIDENCE OF TYPES  
OF PNEUMOCOCCI

	Total Cases	Pneumo- coccus Present	Per- centage Showing Pneumo- coccus	Type 1	Type 2	Type 2a	Type 3	Type 4
Normals.....	46	10	21.7	0	0	1	0	9
Colds								
Acute rhinitis.....	47	17	36.2	0	1	2	3	11
Acute pharyngitis.....	9	1	11.1	0	0	1	0	0
Acute rhinitis and bronchitis.....	11	5	45.5	1	1	1	0	2
Acute pharyngitis and bron- chitis.....	4	2	50.0	0	0	0	1	1
Tonsillitis.....	6	2	33.3	0	0	0	0	2
Incidence of pneumococci in all colds.....	77	27	34.9	1	2	4	4	16
Percentage of incidence of types in positive cases of colds.....	..	..	....	3.7	7.4	14.8	14.8	59.2

The results presented in table 1 indicate that the pneumococcus is present in a considerably higher percentage of cases of colds than in the series of normal persons examined under the same conditions. No particular variation in the degree of incidence among the various types of inflammation is evidenced, except that in general it may be observed that pneumococci are found more commonly in those cases which show an involvement of the bronchi, together with an inflammation of the nose or pharynx.

By far the most common group of coccus found in normal persons was the pneumococcus 4. This was also the case with pathologic throats, but in these representatives of all of the types were isolated, even the fixed types 1 and 2 being observed.

No attempt was made to determine the exact number of pneumococci in relation to members of the gram-negative, streptococcus and Pfeiffer bacillus groups. Rough plate readings were made, however, and in general, green-producing colonies constituted from one third to three fourths of the total colonies developing on blood-agar plates, inoculated directly with the swabs from the nasopharynx.

#### A STUDY OF THE PNEUMOCOCCI INVOLVED IN EPIDEMIC SORE THROAT

The cases of acute respiratory infection which have been previously described constitute sporadic cases developing among a typical urban population. In the course of this work, a restricted epidemic of sore throat occurred among the children attending the School of Education of the University of Chicago. The clinical picture of these cases was out of the ordinary. This fact, together with the evident communicability of the infection, attracted attention to the epidemic which apparently was localized in this particular institution. It has been studied intensively by Sharp, Norton and the writer, and a complete report will be given in a later paper. Certain data which are of importance in a general consideration of the relation of the pneumococcus to upper respiratory infections, will be given here briefly.

A series of nine cases was selected from among those first encountered in the epidemic, and examinations were made for a period of approximately three months at weekly or bimonthly intervals. An additional examination was made after the lapse of 4 months, when a recurrence of the pathologic condition developed, following summer vacation.

A type 4 pneumococcus was isolated consistently from all 9 patients throughout the course of the infection and for periods varying from 2 weeks to at least six weeks after all clinical symptoms had disappeared. In several recurrent cases developing after 5 months, in the same institution, pneumococcus 4 was again isolated in most of the cases. Serologic identity of all of these type 4 pneumococci has been proved. This work is described later in the course of this paper.

A number of normal throats, 13 in all, were examined in the course of the study for the presence of pneumococci in order that a control



might be obtained on cases of the infection. The results obtained from the examination of 2 such series, selected at random from approximately the same age group and the same school population, showed that the particular organism observed in the pathologic throats was uniformly absent in the normal throats. Pneumococci were demonstrated in several instances, among them four type 4 and two type 2a pneumococci. These type 4 organisms were, however, proved to be serologically distinct from those involved in the epidemic, with a single exception. The latter instance might well be explained on the basis of the individual representing a normal carrier condition.

#### THE PNEUMOCOCCUS IN INFLUENZA

In the course of our studies on common colds, there occurred in the early months of 1920 a recurrence of the 1918 pandemic of influenza. Work on colds was suspended for the time being, and investigations were confined to a similar study of the incidence of the pneumococcus in influenza. On account of the brief period during which cases were available, only a limited number could be studied. Three separate series were investigated in order that the bacteriology of like conditions in different localities might furnish some basis for comparison and possibly permit a generalization on bacteriologic conditions in this type of infection.

The attention of various investigators had been called to the prevalence of pneumococci in influenza in the epidemic of 1890, and greater or less stress has been laid on its importance in this condition ever since. Even before Pfeiffer<sup>28</sup> called attention to the bacillus which was held for so many years to be the direct etiologic agent in influenza, the pneumococcus had been studied in its relation to the disease. Marmorek<sup>29</sup> was among the first to encounter the pneumococcus in influenza. Weichselbaum,<sup>30</sup> in more searching studies, found the pneumococcus to be among the most frequent invaders. Further evidence concerning the presence of the organism in influenza and its sequelae was furnished by Kirchner,<sup>31</sup> Babes,<sup>32</sup> and Levy.<sup>33</sup> At this time the organism was apparently felt to have no special significance in the direct etiology of the condition. The general inference of all of these workers was essentially that the virus of influenza, whatever it was, merely prepared the way for the entrance of the pneumococcus as a secondary invader.

In the most recent pandemic, that of 1918, a great number of workers found the pneumococcus in cases distributed throughout the country. In certain

<sup>28</sup> Deutsch. med. Wehnschr., 1892, 18, p. 28.

<sup>29</sup> Wien. klin. Wehnschr., 1890, 3, p. 143.

<sup>30</sup> Ibid., p. 104.

<sup>31</sup> Ztschr. f. Hyg. u. Infektionskr. 1890, 9, p. 528.

<sup>32</sup> Centralbl. f. Bakteriöl., 1890, 7, p. 233.

<sup>33</sup> Berl. klin. Wehnschr., 1890, 27, p. 143.

districts it seemed to be the chief organism involved. Lamb and Brannin<sup>34</sup> found it to be among the more important organisms in influenza at Camp Cody. The extended study by Hirsch and McKinney<sup>35</sup> at Camp Grant indicated the pneumococcus to be the chief organism involved in cases occurring at that post during the height of the epidemic. Later studies<sup>36</sup> on postepidemic cases, however, demonstrated a high incidence of hemolytic streptococcus infections, less frequently pneumococcus. In general, one may say that all workers recognized the pneumococcus as constituting one of the three or four more important groups of bacteria concerned in the infection. In some localities, it was the dominant species.

The group of cases included in the present report is derived from three different sources. A number of cases developed among the same general student population at the University of Chicago from which we had been drawing our material for the study of common colds. A group of cases occurring at the Great Lakes Naval Training Station constituted our second series, while the third series was obtained from soldiers stationed at Camp Grant, Rockford, Ill. The results of the examinations from these three series of influenza cases are contained in table 2.

TABLE 2  
THE PNEUMOCOCCUS IN INFLUENZA

	Total Cases	Pneumococcus Present	Percentage Showing Pneumococcus	Type 1	Type 2	Type 2a	Type 3	Type 4
Great Lakes.....	17	6	36	1	3	1	1	0
Camp Grant.....	12	4	33	0	0	0	0	4
University of Chicago.....	8	4	50	0	0	0	1	3
Incidence of pneumococci in all cases.....	37	14	38	1	3	1	2	7
Percentage of incidence of type pneumococcus in positive cases.....	..	..	..	7	21	7	14	50

Although the total number of cases is too small to warrant definite conclusions, the results indicate in a degree that the presence of the fixed types is considerably more common in influenzal conditions than in common colds. It even approaches somewhat the type incidence usually encountered in lobar pneumonias. Pneumococci were not present in influenza cases much more commonly than in common colds. Data on cases of cold showed an incidence of 34%, in influenza, 38%. The organism was present about twice as frequently in influenza as it was in the series of normal persons studied.

<sup>34</sup> Jour. Am. Med. Assn., 1919, 72, p. 1056.

<sup>35</sup> Jour. Infect. Dis., 1919, 24, p. 594.

<sup>36</sup> Ibid., 1919, 25, p. 393.

AN ATTEMPT TO DETERMINE THE SPECIFIC RELATIONSHIP  
OF THE PNEUMOCOCCUS TO COMMON COLDS

Experimental data have been presented which furnish evidence of the presence of the pneumococcus in common colds and influenza, and furthermore indicate that in normal persons these organisms can be found in about 21% or more of cases. It seemed desirable to obtain information bearing on certain fundamental questions. Is the particular pneumococcus which is found in such an acute respiratory infection merely present, more or less accidentally, as a normal inhabitant of the tract at the time of the developing infection? If so, does it evidence any activity, any symbiotic relationship to the infecting bacterium or virus during the course of the infection? If not, does it gain entrance into the inflamed area coincidentally with developing symptoms, previous to that time, or somewhat later in the course of the infection? Does it disappear soon after the subsidence of clinical symptoms, or may it persist for appreciable lengths of time, constituting a carrier condition, permanent or temporary? With these questions in mind, a group of 10 normal persons was selected, and each one was subjected to daily routine cultures over a period of about two and one-half months. Each case was watched closely for the onset of any symptoms which would indicate an oncoming cold. Detailed daily studies of each culture were not made after the initial culture, except when colds developed. But from time to time, at intervals of perhaps two weeks, such examinations were made in order to maintain a general idea of the constitution of the flora. The plates were critically examined each day, however, and a percentage estimate made of the different types of colonies which developed.

During the course of these experiments, 5 of the 10 subjects in the series developed one or more colds, the other five remaining normal throughout the period of observation. Among the persons who maintained a normal condition of the upper respiratory tract, different conditions were observed as regards the pneumococcus.

Subject A-4 gave cultures of the pneumococcus of the type 4 variety on the initial examination, and this organism persisted in subsequent cultures over a period of 2 months, green producing cocci being the predominating organisms in the flora continuously. It then disappeared and further cultures proved negative.

Early plate readings of A-5 showed the absence of green producing cocci in the flora. Fifteen days after the first examination, green

colonies became dominant on the plates. Cultural tests showed that they included pneumococcus 4. They continued in the throat until 2 months after the beginning of the study of the case, when *B. mucosus capsulatus* became dominant, and indeed crowded out the green-producing colonies. Cultures taken after this time yielded no pneumococci.

The group of green-producing cocci was the predominating type of organism in the cultures from A-7 throughout the course of the study. *Pneumococcus* 4 was identified.

Subject A-8 was characterized by the presence, largely, of only 2 of the general groups of respiratory micro-organisms during the period of examination, namely, green-producing cocci and organisms of the *Micrococcus catarrhalis* group. No pneumococci were isolated at any time.

The flora of A-10 was found to be subject to considerable variation. Early in the course of the experiments, the *Micrococcus catarrhalis* group predominated, with the greens numerically next in order. Two months after the first examination, the greens increased markedly in numbers and pneumococci belonging to type 4 were isolated. This condition was only transitory, however, for soon the Pfeiffer bacillus dominated all plates with the pneumococcus no longer capable of being demonstrated in culture.

It would seem, then, that the flora of the normal person, at least during the winter months, is subject to distinct variations in constitution from time to time. One type of organism may dominate in a given respiratory tract for varying lengths of time, to be eventually supplanted by another. In 4 of the 5 cases studied, the pneumococcus was found at one time or another. It persisted in a given throat for as long as 2 months or more, and constituted a distinct carrier condition. Again, it was present in demonstrable numbers for a few days only, with subsequent replacement by other species of bacteria. In these 5 cases the pneumococcus came and went, or persisted indefinitely, without the development of clinical symptoms.

Somewhat more uniform conditions are evidenced among the 5 patients who developed colds in the course of the experiments. Green producing cocci were present in the upper respiratory tract of A-1 from the time of the original culturing, and extending over two months time. Pneumococci were, however, not present, the green-producing cocci being *Streptococcus viridans*. The average incidence varied from 20

to 50% per cent of the total flora, as evidenced by plate cultures. Occasionally green colonies dominated the plates to the practical exclusion of all other forms. Two weeks after initiating cultures, an acute pharyngitis developed. Pneumococci were not demonstrated in the course of the cold. About 2 months after the original culturing, a second acute pharyngitis was manifest. The first day on which symptoms were present no pneumococci were present in cultures, and the percentage of greens had not noticeably increased. The second day's culturing gave the same bacterial picture. On the third day, however, pneumococcus 4 was isolated. It was absent in cultures taken one week later, although slight symptoms of the cold persisted.

Two colds developed in the course of the study of A-2. Symptoms of a beginning rhinitis were present on the second day after study of the case had been initiated. Pneumococci could not be isolated throughout the course of the cold, which lasted 8 days. Three weeks later a second rhinitis developed, and this time a pneumococcus 4 was obtained from cultures taken on the first day that symptoms of the inflammation were present. The organism persisted in cultures taken during the course of the cold but disappeared with the subsidence of the infection.

A-3 likewise developed an acute rhinitis the second day after the beginning of the investigation. Eight complete examinations were made during the course of the infection, but the pneumococcus could not be demonstrated in culture. Six weeks after the first infection, a second rhinitis was observed. A culture was not obtained until the second day after the beginning of the cold, but at that time the pneumococcus 4 was found in large numbers. During the four days on which symptoms were manifest, the organism was demonstrated each day, and it continued in cultures for 10 days thereafter. Following that time it remained negative during the 3 successive observations.

Green-producing cocci were uniformly in the minority in cultures taken from A-6, oftentimes being absent. The gram-negative cocci and staphylococci were the predominating organisms in the flora. Three weeks after the first culture, an acute rhinitis was diagnosed. The Pfeiffer bacillus seemed to be the organism chiefly involved. Pneumococci were not isolated from any of the cultures taken during the study of this case.

The fifth subject who developed a cold while under observation was A-9. Green-producing cocci were consistently the predominating type in

cultures, usually constituting about 90% of the colonies developing on blood-agar plates, but no pneumococci could be determined. Three weeks after the initiation of the study, a slight rhinitis followed. A type 4 pneumococcus was cultivated on the first day that symptoms appeared, but it disappeared from cultures soon, and subsequent cultures were negative for the organism.

Analysis of the last 5 cases brings out certain facts. Certainly the pneumococcus was not involved in the etiology of all of the colds developing in the series. The first colds of subjects A-2 and A-3 could not be connected in any way with an etiology involving that organism; nor could the cold which was observed in A-6. The second colds occurring in both A-2 and A-3, as well as the rhinitis which was observed in A-9, may have been of pneumococcal origin. In these 3 cases of rhinitis, the pneumococcus was present in cultures coincidently with the onset of symptoms. As regards the second cold of A-1, the question is problematical, but most likely the pneumococcus isolated in that instance can best be interpreted as a secondary invader. One cannot, however, definitely ascribe a pneumococcal origin to even these 3 cases, for there is no direct knowledge of the period of incubation of such infections. The presence of the pneumococcus at the time of infection may have meant merely that under the stimulus of the exciting agent of the cold, pneumococci normally in the throat, although present in such small numbers that they could not be demonstrated, rapidly began to multiply, and possibly acquired as well a distinct pathogenicity. Thus one cannot determine whether the presence of the organism indicates direct etiology or symbiotic relationship to the infection.

From the study of this series of cases, then, one is led to conclude that intermittently normal persons may develop either a temporary or even chronic carrier condition for Fränkel's pneumococcus, without the organism causing any symptoms of pharyngeal or nasal inflammation. A certain percentage of colds may be due to the invasion of the respiratory tract by the pneumococcus since, in the cases cited, pneumococci have been demonstrated not to be present in the normal condition of the nose and throat. Furthermore, evidence of a beginning inflammation was present coincidently with their determination in culture. In other circumstances it appears that the organism invades the inflamed area secondarily to some other organism which has primarily incited the infection.

THE EXTENT OF THE CARRIER CONDITION FOR THE  
PNEUMOCOCCUS

During the course of the study of the "A" series of cases previously described, it was observed that a given pneumococcus was carried in the throats of persons a relatively short time following convalescence from these simple types of respiratory involvement, acute rhinitis and pharyngitis. Ten days was the longest time observed in the cases studied. These few cases would indicate that there is little danger of spread of contagion from the convalescent.

In the cases of epidemic tonsillitis and pharyngitis described previously, the School of Education Series, the convalescents maintained the carrier condition for a much longer time. The majority of the 9 patients in this series revealed the pneumococcus in examinations following the infection for at least 6 weeks, most of them 2 months or longer, while in 3 patients it persisted for at least 7 months. It must be remembered, however, that this was an unusual type of respiratory involvement and, moreover, the particular pneumococcus 4 was distinct from any other subgroup of pneumococcus 4 isolated in the course of our studies on colds.

Attention was directed to the possibility of a certain percentage of normal persons constituting a population of permanent pneumococcus carriers. A series of normal persons was studied in order to ascertain whether the carrier state as observed in normal persons was transitory in type or was as a rule long continued. Six subjects were examined during the spring months, April and May, while a second group of 5 were studied intensively during the early winter months, Nov., Dec., and Jan. Four cases of the Spring series, when examined at weekly intervals, did not at any time give evidence of pneumococci of any type. Two cases did present pneumococci at some time during the experiments. One subject, C623, was positive at the first examination, and continued to give cultures of type 4 pneumococcus for three weeks, after which the cultures were negative. The second subject, C492, proved negative for pneumococcus at the first examination but one week later cultures were positive. Cultures were still positive about 7 weeks later when conditions prevented further study.

Of the series of 5 normal cases studied during the early winter months, 4 at some time or other picked up the organism, although all were initially negative. A temporary carrier condition developed.

respectively, for periods of 2 months, 2 months, 1 week, and 3 weeks, in the 4 cases.

The limited number of cases studied forbids definite conclusions. Indications are that protracted carrier condition of the pneumococcus is rare but that temporary carriers develop with considerable frequency, probably more commonly in winter months than during the spring and summer. Based on the cases studied, the average time of the carrier state is about one month.

SEROLOGIC STUDY OF TYPE 4 CULTURES TAKEN FROM PERSONS  
WITH COLDS, FROM NORMAL PERSONS AND FROM PERSONS  
WITH INFLUENZA

Pneumococcus 4 has been by far the most common pneumococcus observed in our study of acute respiratory infections. Previous studies cited have shown a decided variation in the relation of any one strain of the type 4 pneumococcus to another, and indeed the group is recognized as a collection of heterologous strains having little serologic relationship. So common, however, was its occurrence in our cases of various clinical type, that if any particular organism, for example the pneumococcus, was to be given any weight in a determination of a common etiology, some specific relationship necessarily had to be demonstrated between these various strains. In other words, was a single variety of pneumococcus 4 involved, or did the various strains differ in biologic properties?

Fifteen strains were selected from type 4 pneumococci isolated from various types of acute respiratory involvement and from normal persons. These 15 cultures, representing type 4 pneumococci present in normal persons, in cases of influenza, and in various types of common colds, have been studied intensively.

Monovalent rabbit serums were prepared for these 15 strains by injecting the animals with cultures of pneumococci grown on blood-agar slants and washed off in salt solution. Killed cultures were used in the early injections and were given intravenously for 3 successive days, followed by a like period of rest. Later live cultures were employed. As a general rule, about 20 inoculations were required to obtain a relatively high agglutination titer for the coccus. All serums so prepared, agglutinated the homologous strain in a dilution of at least 1:600, some as high as 1:1,600. After a sufficiently high titer had been obtained, the rabbits were bled to death, and the serum preserved in



frozen ampoules. Serum so preserved will not show a decided drop in agglutinating power for some time. One serum, stored for 9 months, had lost only 30% of its agglutinating value.

All 15 strains were tested by agglutination with each of the immune serums so prepared. The agglutinating titer for the homologous strain, as well as cross agglutination with other strains, was determined for each serum. The tests were made by adding 1 c.c. of a young broth culture of pneumococci to an equal volume of diluted serum. Control tests included monovalent horse serums for types 1, 2 and 3, in dilutions of 1:40, 1:40 and 1:20, respectively, a normal rabbit serum control, in a final dilution of 1:25, and a saline control. The tests were incubated at 37 C. for 2 hours, preliminary readings made, and the tubes placed in the icebox over night. Final results were recorded from readings made the following morning.

TABLE 3  
CROSS AGGLUTINATION OF TYPE 4 PNEUMOCOCCI \*

Cul- ture	Source	Monovalent Rabbit Serums														
		C623-4	C492-7	E-24	E-27	G4	G6	C627	C628	C230	C201	C163	C504	C548	C235	C114
C623-4	Normal.....	800	—	—	—	—	—	—	—	—	—	50	—	—	—	—
C492-7	Normal.....	—	1200	—	—	—	—	—	—	—	—	—	—	—	—	—
E-24	Normal.....	—	—	800	—	—	—	—	—	—	—	—	—	—	—	—
E-27	Normal.....	—	—	—	800	—	—	—	—	—	—	—	—	—	—	—
G-4	Influenza.....	—	—	—	—	1000	200	—	—	—	400	—	—	—	—	—
G-6	Influenza.....	—	—	—	—	300	1200	—	—	—	50	—	—	—	—	—
C 627	Influenza.....	—	—	—	—	—	—	1600	—	—	—	—	—	—	—	—
C 628	Influenza.....	—	—	—	—	—	—	—	600	—	—	—	—	—	—	—
C 230	Acute rhin- itis.....	—	—	—	—	—	—	—	—	1000	—	—	200	—	—	—
C 201	Acute rhin- itis.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C 163	Acute rhin- itis and bronchitis..	25	25	—	—	400	200	—	—	—	800	—	25	100	—	25
C 504	Acute rhin- itis.....	—	—	—	—	—	—	—	—	—	—	1000	—	—	400	—
C 548	Acute rhin- itis.....	100	—	—	—	—	—	—	—	—	—	—	500	—	—	—
C 235	Tonsillitis...	—	—	—	—	—	—	—	—	300	—	—	—	600	—	—
C 114	Acute rhin- itis.....	25	—	—	—	—	—	—	—	—	200	—	—	25	800	—
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	1200

\* Figures represent highest agglutinating titer; — = absence of agglutination.

Control tests were all negative with the exception that strain C201 was agglutinated by type 1, 2 and 3 monovalent horse serums. The normal rabbit serum and saline controls for this strain were negative.

The cross agglutination experiments indicate common properties peculiar to certain of the organisms studied. Final group classification and identity of strains was proved by the use of the agglutinin absorption technic. Those serums which showed agglutination with strains

other than the specific organism used in its preparation, were absorbed by the cross agglutinating organism, and the absorbed serum then tested by agglutination for the persistence of agglutinins for the homologous pneumococcus.

The technic of agglutinin absorption was as follows, essentially that used by Avery<sup>16</sup> in his study of atypical type 2 strains. The washed bacterial residue from 150 cc of an 18-24 hour old broth culture was added to the undiluted serum. Before adding the organisms, they were killed by heating to 55 C. for one-half hour. The mixtures were incubated at 37 C. for 2 hours with frequent agitation, and then were placed in the icebox over night. The serum was then freed of bacteria by centrifugation, and the supernatant fluid pipetted off. This absorbed serum was used for agglutination tests with the homologous organism for the presence or absence of agglutinins. The technic was controlled by the absorption of the serum by the homologous organism as well, with agglutination tests following, in order to assure that the amount of bacterial residue used was sufficient to remove all agglutinins.

By these methods it was determined that certain of these type 4 strains fell into restricted groups. Strains G4 and G6 from the Camp Grant epidemic of influenza and strain C201 from a case of rhinitis in the Chicago series proved to be allied. Contact between the three patients was unlikely with the exception of the 2 patients with influenza. Likewise, strains C163 and C235, isolated respectfully from cases of acute rhinitis and bronchitis, and from acute tonsillitis, were found to constitute a second small group. Strains C230 and C548 also possessed like serologic properties. No relationship could be established between C504, a rhinitis strain and C623-4, a normal strain, although cross agglutination had occurred. Agglutination had been due apparently to minor agglutinins. Strains C492-7, C623-4, E24, E27, C627, C628, C504 and C114, all differed in biologic properties.

Immunologic experiments on the predominating type of pneumococcus isolated in the course of this study show that there is little relationship between such strains encountered in various acute respiratory conditions. Of the 15 studied, 8 were entirely distinct one from the other. Grouping could be effected in 3 instances, one group including 3 of the strains studied, the others 2 each. It seems certain that there is little relationship between the type 4 pneumococci encountered in the acute infections of the upper respiratory tract. In the light of studies made on type 4 pneumococci occurring in lobar pneumonia, this was logically to be expected.

Distinctly different results have been obtained in a study of the group 4 strains obtained from those cases of epidemic tonsillitis and pharyngitis previously described, which occurred in the School of Education at the University. Common agglutinating properties were found to exist between all strains isolated from the original 9 cases studied, as well as from 2 others observed later. A monovalent rabbit serum prepared from strain E4-6 agglutinated all of the strains mentioned in the foregoing. Cross agglutination was not obtained with type 4 strains from other respiratory infections or from normal sources. Control tests with the fixed types were likewise negative. Serologic identity of all these strains was thus established.

In general, then, one may conclude that the pneumococci which are encountered in various types of respiratory involvement are unrelated. There is no common strain which is involved in the etiology of these infections, and no specific etiology may be claimed for the pneumococcus. In isolated instances, however, such as the epidemic at the School of Education, a common strain of pneumococcus may be involved in a given group of cases.

#### THE COMPARATIVE VIRULENCE OF PNEUMOCOCCI ISOLATED FROM NORMAL THROATS AND FROM THROATS AFFECTED BY ACUTE RESPIRATORY CONDITIONS

The observation was made during the study of material from various sources by the mouse method that a difference in degree of pathogenicity for these animals was apparent. Cultures from normal throats which were later proved to contain pneumococci had little effect and indeed rarely produced death of the animal within 2 or 3 days. Cultures from patients with cold differed. A few would cause death of the mice in 24 hours or thereabouts, but most of them had a decidedly lesser effect. During the study of influenza cases it was apparent that the organisms involved were much more virulent for mice.

Of course the original injections given the animals contained a variety of organisms besides the pneumococcus, and the variation in lethal effect therefore could not definitely ascribed to a difference of virulence among the pneumococci themselves. For this reason a comparative study of pure strains of pneumococci from various sources has been made. It is well known that the virulence of the pneumococcus varies greatly according to the length of time it has been carried in artificial culture. It gradually decreases in virulence. Likewise its

virulence for a given species can be enhanced by frequent passage through that animal. In order that results might be directly comparable, all of the tests for virulence have been made by using blood-agar cultures having the same history. These pneumococci, after removal from the throat, had undergone one mouse passage, had been cultivated on blood-agar plates and then picked to blood-agar slants. They were, then, in the second generation on artificial medium after a single mouse passage. By this method, conditions were exactly comparable and, furthermore, tests for virulence were made within the shortest possible time following removal of the organism from the throat.

In obtaining cultures for inoculation, care was taken to seed as nearly as possible the same extent of blood-agar medium. Growths were washed off in 5 c c of warm sterile broth, and appropriate dilutions made in broth so that the final inoculum was contained in 0.5 c c volume. Mice were inoculated at once intraperitoneally. The results obtained with cultures from normal persons, from patients with colds and from patients with influenza are given in table 4.

TABLE 4  
COMPARATIVE VIRULENCE FOR MICE OF PNEUMOCOCCI ISOLATED FROM VARIOUS SOURCES \*

Culture	Source	Type of Pneumococcus	Amount of 24-Hour Blood-Agar Slant			
			0.01	0.001	0.0001	0.00001
C 623-4	Normal.....	IV	S	S	—	—
C 492-7	Normal.....	IV	S	S	—	—
E 22	Normal.....	IV	S	S	—	—
E 28	Normal.....	IV	S	S	—	—
G 4	Influenza.....	IV	D14	D15	D30	S
G 6	Influenza.....	IV	D14	D48	S	S
C 504	Acute rhinitis.....	IV	D14	D48	S	S
C 548	Acute rhinitis.....	IV	S	S	S	S
GL 10	Influenza.....	II	D14	D13	D14	D30
GL 12	Influenza.....	II	D12	D14	D14	D22
C 230	Acute rhinitis.....	IV	D48	S	S	S
C 201	Acute rhinitis.....	IV	D30	S	S	S
C 163	Acute rhinitis and bronchitis	IV	D12	D36	S	S

\* S = survived 72 hours; — = not tested; figures represent number of hours before death.

Pneumococci from normal throats uniformly failed to cause death of the mice within the period of observation, even with the largest doses used. Cultures derived from patients with influenza were considerably more virulent than those from normal persons, even in comparison with the same group of organism, type 4. Likewise cultures from persons with colds possessed a higher degree of virulence than type 4 cultures from normal throats.

## DISCUSSION

Pneumococci are observed in various simple inflammations of the upper respiratory mucous membrane, grouped together under the general term of "common colds," somewhat more frequently than in throats which do not show lesions, although the increased percentage incidence to the particular region affected, namely rhinitis, pharyngitis, tonsillitis to the particular region affected, namely rhinitis, pharyngitis, tonsillitis or combinations of these conditions with bronchial involvement, do not show any appreciable degree of difference in the frequency with which pneumococci are encountered. If any conclusions may be drawn from the limited number of cases studied, it would appear that the organism is somewhat more common in infections which include involvement of the bronchi.

In patients with influenza, pneumococci are frequently encountered, but again in numbers not much greater than in normal persons, and slightly increased over the incidence in common colds. The frequent occurrence of fixed types is chiefly of interest in comparison with the types of pneumococci found in common colds and in normal persons.

While the patient with the average common cold or influenza will only show the presence of the pneumococcus in somewhat greater frequency than the ratio of 1:3, nevertheless outbreaks of respiratory infection may occur in which this particular coccus is involved in practically all cases. Such an instance was demonstrated in our School of Education series.

By observing the bacterial changes evidenced in oncoming colds, it was found that in certain instances pneumococci appear in the throat practically at the same time that developing symptoms are observed. Since there is no exact knowledge of the period of incubation in common colds, one can merely conjecture as to the significance of the pneumococci present. If common colds present the relatively short incubation period which we know is characteristic of influenza, then it would seem that in some cases this organism may be the direct causative factor. In other instances, the presence of the pneumococcus in the inflamed area was demonstrated relatively late in the course of the infection. It would seem to be present in such cases as a secondary invader.

Serologic studies of pneumococci, involving type determinations of the organisms from pathologic throats and a more careful study of the predominating group, type 4, give evidence that no one variety of

pneumococcus is concerned in those cases in which the organism is present in one capacity or another.

Interesting information is furnished by the study of the relative virulence of pneumococci from various respiratory infections and from normal sources in respect to their relationship to disease. Our early individual case studies and the results obtained in our "A" series in which developing colds were observed both pointed to the conclusion that the pneumococcus certainly could not be involved in the etiology of all colds. The serologic study of various strains obtained from those cases in which pneumococci were found led to the definite conclusion that no common etiology can be proved, for the strains vary decidedly in biologic properties. What, then, is the province of the pneumococcus in these infections, or has it really no significance? Some light is cast on the problem by the virulence studies. Pneumococci from cold sources are surely more virulent for mice than are like cultures derived from normal persons. In other epidemics, such as influenza, it shows a still more heightened virulence.

Granting the fact that the pneumococcus may only play a primary etiologic rôle in a small percentage of cases, as indicated by the studies of our "A" series, still it would seem that the pneumococcus may rather commonly be a secondary invader; or, if present normally in the throat in numbers too small for detection, may increase rapidly in numbers, and under the stimulus of the conditions generated by the inflammation, or a symbiosis with the infecting virus, acquire an increased virulence. It seems probable that in a considerable percentage of upper respiratory infections the pneumococcus is in reality pathogenic, and exercises a real influence in the course of the infection.

#### CONCLUSIONS

The average incidence of the pneumococcus in normal throats, in the present series of cases is about 21%. Cases of common colds showed pneumococci more commonly, 35%, and the same was true of influenza, 38%.

Type incidence of pneumococci in normal persons and in persons with colds was characterized by the infrequent occurrence of the fixed types of pneumococci. Fixed types are somewhat more frequent in influenza than in other acute infections of the upper respiratory tract.

No serologic relationship could in general be demonstrated between the pneumococci found in these infections. Types other than 4 were

observed. Type 4 strains in this series show a division into 8 different strains and 3 small groups. No common strain of pneumococcus was present in acute respiratory infections.

A particular group of cases occurring in a localized epidemic showed a uniform occurrence of the pneumococcus in practically all of the cases studied, a type 4 pneumococcus. The strains were proved to be serologically identical. It seems certain, then, that although the general statement can be made that pneumococci are not found in the majority of colds, and that when they do occur they are rarely related to each other, nevertheless instances occur in which a single type of pneumococcus is involved in all cases of a given group.

Comparative virulence tests showed that the pneumococci from colds and from influenza were more pathogenic than the strains from normal throats.

# THE GRAM-NEGATIVE COCCI IN "COLDS" AND INFLUENZA \*

## INFLUENZA STUDIES VII

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No affection is more common in temperate zones, probably, than that group of conditions, varying largely in clinical type and manifestations, which are classed together as "common colds." Relatively little investigation has, however, been directed toward the determination of their etiology. Their usual mild course and the absence of a fatal termination doubtless account for this fact. The tremendous economic loss caused by the incapacitation for work, from time to time, of a considerable proportion of the population, gives them an importance not always recognized. The reduced resistance following colds is likewise important in that it often predisposes to more severe infections, not alone confined to the respiratory tract. It would seem desirable to obtain more exact knowledge of the cause and mode of spread of common colds and influenza.

The varied flora of the upper respiratory tract, under both normal and pathologic conditions makes the problem of the bacteriologic investigation a most complicated one. Recent studies by Bloomfield<sup>1</sup> have contributed materially to our knowledge of the bacteria which are normally present in the throat, and to the conditions which govern invasion by organisms not commonly present. Some circumstance other than the mere presence of a given bacterium seems to be necessary for the initiation of disease. Large amounts of bacterial growth, from cultures of various organisms, *Sarcina lutea*, *Staphylococcus albus*, *B. coli*<sup>2</sup> *B. influenzae* of Pfeiffer,<sup>3</sup> *B. mucosus capsulatus* of Friedländer<sup>4</sup> and *Streptococcus hemolyticus*,<sup>1</sup> were smeared on the tongue, pharynx, nasal septum and into the tonsillar crypts of persons free from unusual abnormalities of the upper air passages. The various organisms disappeared as a rule within 24 hours. In no case was any demonstrable lesion or general reaction set up. The mechanical flushing action of the secretions seems undoubtedly the most important element in the disposal of bacteria introduced into the

Received for publication June 3, 1921.

\* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York. Part of the expense of these studies has been met by a grant from the University of Chicago.

<sup>1</sup> Am. Rev. Tuberc., 1920, 4, p. 247.

<sup>2</sup> Bull. Johns Hopkins Hosp., 1919, 30, p. 317.

<sup>3</sup> Bloomfield, A. L.: Ibid., 1920, 31, p. 85.

<sup>4</sup> Bloomfield, A. L.: Ibid., p. 203; 1921, 32, p. 10.



mouth and nose. The saliva in many instances appears to be an unfavorable medium for bacterial growth.<sup>5</sup> The outstanding fact seems to be that the "normal" intact mucous membranes of the upper air passages are not only impervious to the attack of certain pathogenic bacteria, but that these organisms also fail to colonize on such surfaces. Bloomfield tentatively concludes that there are certain bacteria which constitute a true mouth flora in the sense that they live and multiply and are almost constantly present in most people and persist from day to day over considerable periods of time. Another group of organisms may be occasionally recovered from the mouth, but as a rule they are present for only short periods of time. They seem to be bacteria which are essentially transients and fail to colonize or to survive on normal mucous membranes. Occasional individuals are encountered who "carry" over considerable periods of time organisms which do not belong to the normal true mouth flora.

The micro-organisms which have been studied in greatest detail in their relation to colds and to influenza are the pneumococcus, the varieties of streptococci, and the Pfeiffer bacillus. In addition to these three major groups, there are commonly present in infections of this class, a large group of cocci which are decolorized by the Gram method. Some of these are conceded to be saprophytic inhabitants of the normal nose and throat. Others are regarded as more or less foreign to the normal mucosa and ordinarily present only in pathologic processes.

Studies of the meningococcus during periods of epidemic meningitis have furnished a wealth of information concerning the gram-negative coccal flora of the nose and throat in that infection. The first extended investigation was conducted by von Lingelsheim<sup>6</sup> who described various species of gram-negative cocci, including the meningococcus. Elser and Huntoon<sup>7</sup> report at length on the gram-negative cocci observed in epidemic meningitis. Added observations have been contributed by Dunham.<sup>8</sup> Certain general groups have been reported by all of these workers. The various species, then, which might be expected in such a study as the present one, would include rather rarely the meningococcus, more commonly *M. catarrhalis* and a large group of chromogenic cocci, and in addition certain organisms such as *M. pharyngis siccus*, *Diplococcus crassus* and *diplococcus mucosus*, of less frequent occurrence.

The study of this group of gram-negative cocci, particularly *M. catarrhalis*, in their relation to the etiology of common colds and influenza, forms the basis of the work here reported.

Two general groups of acute respiratory infection have been investigated. The first included a number of common colds of varied clinical type, occurring in an urban population. The second group was composed of cases from a recurrent epidemic of influenza, developing in the early winter months of 1920, and characterized by the same general clinical picture which was manifested in the pandemic of 1918.

<sup>5</sup> Ibid., 1920, 31, p. 118.

<sup>6</sup> *Klinisches Jahrbuch*, 1906, 15, p. 373.

<sup>7</sup> *Jour. Med. Research*, 1909, 20, p. 380.

<sup>8</sup> *Jour. Infect. Dis., Suppl. 2*, 1905, p. 10.

It was decided in the preliminary experiments, to determine whether or not there was any decided increased incidence of gram-negative cocci in respiratory infections, over the occurrence in the normal throat. Furthermore, was any particular kind of gram-negative coccus found more commonly in pathologic than in normal throats? By this general procedure it was hoped to acquire a certain amount of information bearing on the rôle of *M. catarrhalis* and the gram-negative group in common colds and influenza.

Mere demonstration of an increased incidence of a given organism in respiratory infections can, however, simply furnish a clue to its significance. Such an organism may have been present as a normal inhabitant of the mucous membrane at the time of the developing cold, and be involved in no way in the infection. It may, although normally there, be playing an accessory rôle in the inflammation. Again, it may be present as a secondary invader to the bacterium or virus primarily causing the infection. Finally, it may be the true etiologic agent. Experiments involving a determination of the progressive bacterial changes in oncoming colds can furnish valuable information on these points.

#### METHODS OF STUDYING THE BACTERIAL FLORA IN UPPER RESPIRATORY INFECTIONS

Two general sources of material may be taken as available in a study of this nature. Sputum is assuredly preferred in a study of the bacterial flora of the pneumonias, and might be considered as desirable in studies of the simpler infections of the respiratory tract which present bronchial involvement. Involvement of the bronchi is, however, the exception rather than the rule in the usual type of "common cold." Even when present, the sputum raised is at best scanty and irregular. Consequently, the study of the bacteriology of all upper respiratory infections has been confined to examination of material obtained by swabbing the area of infected mucous membrane.

It seemed logical to suppose that the infecting organism in a given inflammation, whether it be a gram-negative coccus or any organism, would be present in greatest numbers on the mucous membrane first affected. Physical findings were determined by another member of the staff, Dr. W. B. Sharp, to whom I am indebted for all clinical data contained in this report. Depending on the diagnosis made, certain areas were subsequently swabbed. Early in the course of the experiments it became evident that gram-negative cocci were of extremely

common occurrence in the nasopharynx. Later studies have confirmed this observation. Of the first 75 cases in which gram-negative cocci were found, 65 gave cultures from the nasopharynx. An exact record was kept of the region from which each strain of gram-negative coccus was isolated. Twenty-five cases showing gram-negative cocci, were swabbed from the nasopharynx and tonsillar regions. Nineteen of the nasopharynx cultures were positive while 20 from the posterior pharynx contained gram-negative cocci. These cultures were chiefly derived from cases of pharyngitis and tonsillitis, sometimes accompanied by bronchial involvement. Another series of cases were subjected to duplicate swabbings from the nose and nasopharynx. Of 50 cases in this group in which gram-negative cocci were demonstrated, 46 gave cultures from the nasopharynx against 30 from the nasal cultures.

The region of the nasopharynx is the chief site for localization of gram-negative cocci in the pathologic upper respiratory tract. Next to the nasopharynx, they are found with greatest frequency in the tonsillar region, with the anterior nares showing the lowest incidence. On the basis of this preliminary work, a routine procedure was adopted for study of all colds. Cases diagnosed as rhinitis were swabbed from the middle fossa and floor of the nostril and from the nasopharynx while all other inflammations, those of pharynx, larynx, tonsils or combinations of these cases with bronchial infection were swabbed from the nasopharynx and from the tonsillar region. Patients with cases of influenza were likewise swabbed in the nasopharynx and pharynx. Ordinary wooden applicators with sterile cotton swabs were used for the nasal and tonsillar swabs, while the bent wire with cotton swab, as introduced by Mathers<sup>9</sup> was employed for obtaining nasopharyngeal material. Particular care was taken in making nasopharyngeal and tonsillar swabs to avoid salivary contamination.

*Methods of Culture.*—Three different mediums were employed at the beginning of the work, the vitamin-blood-pour-agar plates of Park and Williams,<sup>10</sup> the oleate-hemoglobin-agar of Avery<sup>11</sup> and a 5% sheep-blood agar, made from a veal infusion base, with 2% agar added, and the reaction adjusted to  $P_H$  7.8. The vitamin blood medium has been suggested as a general enriching medium and especially favorable to growth of the meningococcus. It seemed a highly

<sup>9</sup> Ibid., 1918, 22, p. 523.

<sup>10</sup> Amer. Jour. Publ. Health, 1919, 9, p. 45.

<sup>11</sup> Jour. Am. Med. Assn., 1918, 71, p. 2050.

desirable medium for culture of all gram-negative cocci. The oleate-hemoglobin medium was developed late in the course of the recent pandemic of influenza, and has been found by numerous workers to be especially suited to the culture not alone of the Pfeiffer bacillus, but of all gram-negative organisms. Plain veal infusion blood agar has been so universally suited to cultivation of organisms from respiratory conditions that it was included as the third medium.

Careful records were kept of the results obtained from the use of each of the three mediums, during the early part of the work, that their comparative value might be determined. Not only was the incidence of the various kinds of gram-negative cocci determined, but a rough percentage estimate was made of the number of colonies developing, as compared with other groups of respiratory organisms. Of the first 75 cultures, which by one medium or another gave gram-negative cocci, it was found that growths on the oleate hemaglobin plates were positive in 66 instances. The veal infusion blood agar was almost as satisfactory, in that 55 of the cases gave positive cultures on this medium. The vitamine-blood-pour plates left much to be desired. Only 32 of 75 cases, proved by other methods to include these gram-negative organisms among their flora, were positive when cultures were made on this medium. Oleate-hemoglobin agar seems to be the medium best suited for growth of gram-negative organisms from the nose and throat, although plain blood agar is almost as satisfactory. On the basis of these results, the vitamine medium was dispensed with in later work, but cultures were still made on both blood agar and oleate-hemoglobin agar, as it was felt that the factor of using an increased number of plates would in itself tend to give higher percentage findings of the organism for which we were looking. At least 4 plates, and in the early work 6 plates, have been inoculated with material from each case studied. Swabs taken from 2 areas were rubbed over a small area on the surface of the plated medium. The inoculations were then streaked with a looped needle in "sunburst" fashion. Incubation followed at 37 C. for 24 hours. Special care was taken to maintain a high moisture content in the incubator, since study of meningococci has demonstrated the decided importance of this factor in the growth of gram-negative cocci.

The plates were examined under a dissecting microscope for the appearance of characteristic gram-negative-like colonies. These colonies were fished to blood agar slants made by the same formula as the

medium employed for blood-agar plates. An average of 10 colonies were fished to slants from the plates of each case, although no attempt was made to limit the number and oftentimes more were taken.

Colonies characteristic of the gram-negative cocci are readily distinguished on blood-agar medium from other types of respiratory organisms. One kind of gram-negative colony is typically that of the meningococcus. It presents a characteristic small, transparent bluish white colony which is regular in outline and of a watery consistency. The usual catarrhalis-like colony grows somewhat more luxuriantly than that of the meningococcus. Under the microscope the colony appears darker, more compact and has rather ragged margins. It has a granular appearance, especially toward the center of the colony, is semitransparent and causes no change of the medium. A third general type of colony characteristic of gram-negative cocci is one slightly smaller than the meningococcus-like colony and readily differentiated by its consistence. The colony is firm and adherent to the surface of the medium. So firm is the colony that it can readily be lifted with the needle and turned over bodily. Diphtheroid colonies are distinguished from the gram-negative-coccus colonies, with difficulty. Determination of morphology furnishes the only sure method. Staphylococci are readily differentiated by their larger size and distinct white appearance. Colonies of streptococci and pneumococci with their attendant change in appearance of the medium furnish no difficulty, nor do the colonies of the Pfeiffer bacillus.

Few colonies other than those of gram-negative organisms develop on the oleate-hemoglobin agar. This fact, together with the growth favoring qualities which it possesses, makes it a valuable medium. Occasional colonies of diphtheroids may be confused with those of gram-negative cocci. Staphylococci are commonly present, but are distinguishable by their larger size and white appearance.

After 24 hours' incubation the blood agar slant cultures picked from plates were definitely classified as belonging to the group of gram-negative cocci by morphologic examination. The Sterling<sup>12</sup> modification of the gram-stain has been used throughout the work. The gentian violet stain was applied for 10 seconds, the iodine solution for 20 seconds, followed by alcohol. Ten per cent. aqueous saffranin solution, applied for 1 minute, has been used as a counter stain. With each new lot of stain prepared, control preparations were made, to

<sup>12</sup> Monograph Rockefeller Inst. Med. Research, 1917, 7, p. 20.

insure proper differentiating value. A culture of a known meningococcus was employed as a negative control, a culture of *Staphylococcus aureus* as a positive control.

Cultures distinguished on the basis of morphology and Gram reaction as members of the gram-negative coccus group were reserved for classification and further study.

Vedder's starch medium was found to be very well suited for carrying stock cultures of the various gram-negative cocci. Practically all strains encountered grew readily on this medium, especially after a few generations. A few were refractory and stock cultures in these instances were maintained on blood agar until growth could be initiated on starch agar. Freshly isolated cultures were transferred at weekly intervals for the first two or three generations, after which monthly transfers sufficed. Every third or fourth transfer of stock was put onto blood agar instead of starch agar, and after a week retransferred to starch agar. It is a matter of common experience that stock cultures of these organisms are maintained with difficulty, for occasionally a strain will die out, even after long artificial cultivation and for no apparent reason. By these methods, however, unusually good results have been obtained.

#### THE CLASSIFICATION OF GRAM-NEGATIVE COCCI OF THE NOSE AND THROAT

The essential purpose of this investigation has been a classification of the gram-negative cocci which occur in the normal and pathologic upper respiratory tract. Having effected a classification, the incidence of the different groups in various types of common colds and in influenza will be discussed, and an attempt made to determine their probable relation to the given infection.

Primary classification has been made on the basis of reaction toward various carbohydrates. General laboratory practice has favored the use of liquid carbohydrate mediums in the classification of bacteria by fermentation reactions. In our work, however, a solid medium has been employed, and has been found to be much more satisfactory and more easily controlled. Liquid medium is preferable in a study of organisms which give fermentation with production of gas, in that roughly quantitative measurements of the gas may be made. None of the gram-negative cocci heretofore described possess this property. The only other advantage possessed by a liquid medium is the fact that

the degree of acidity may be measured in a broth medium by a determination of the hydrogen-ion value. This may likewise be done within rough limits for a solid medium, by preparing samples of different  $P_H$  value, and observing color values with a standard indicator. There seems then to be no decided disadvantage possessed by a solid medium in a study of this particular group, and its advantages are material. Many of the gram-negative cocci grow with difficulty in broth media. On solid medium the growth is much more satisfactory. Growth in broth can often only be determined by stains and morphological examination. On solid medium growth is readily determined. A large proportion of these organisms, furthermore, do not produce acid from any carbohydrate, and in liquid medium, especially if the broth be somewhat turbid, morphologic examination must frequently be made to insure conclusive readings. Probably the most distinct advantage possessed by an agar-base medium is the control furnished of purity of growth of the inoculated strain, and freedom from contamination during the technical procedure of transferring. Growth characteristics readily demonstrate contaminations on solid medium. Only examination of the morphology will serve definitely to control broth cultures. For these reasons, it was felt that use of medium involving an agar base would not only facilitate technical operations, but would also guard against erroneous results.

The carbohydrate mediums have been made according to the following formula: One pound of finely chopped lean veal is infused with 1 liter of distilled water for 24 hours on ice. At the end of this period the liquid portion is removed by use of a meat press and the albuminoid substances coagulated by bringing to the boiling point. The infusion is then filtered, and the total volume brought up to 1 liter. To this veal infusion 0.5 % sodium chloride, 1 % peptone and 2 % agar are added and the substances brought into suspension. The reaction is adjusted to  $P_H$  7.8 and 1 % Andrade solution<sup>13</sup> added as an indicator. Sugar solutions previously prepared in distilled water were added to give a final concentration of 1 %. The medium was then steamed for 3 successive days in the Arnold sterilizer. It was then slanted, care being taken to leave at least an inch of solid medium in the butt of the tube below the sloped surface. The medium was incubated for 24 hours at 37 C. for sterility. Each lot of medium was inoculated with a known fermenting organism to insure proper reaction.

<sup>13</sup> Jour. Infect. Dis., 1914, 15, p. 227.

Both stab and streak inoculations were made on the several carbohydrate mediums. It is well recognized that growth of certain of the gram-negative cocci is enhanced by a partial reduction in oxygen tension, and conditions best favoring growth were found as a rule to prevail at the butt of the tube, although surface growths were uniformly good. In some few instances growth could not be initiated, but it was found that by smearing the surface of the agar with a small amount of sterile, inactivated sheep serum that growth could always be obtained. This technic was employed for such strains.

Purity of growth in cultures was insured by making pour plates of all cultures just before they were tested for fermentation. Single colonies were fished from the plates to agar slants and examined for morphology. The medium was made in large amounts, and so far as possible strains were tested for acid production in considerable numbers at one time in order that comparable conditions of temperature, moisture and the same lot of medium might prevail.

Cultures were given an initial incubation of 24 hours at 37 C., and preliminary observations were recorded. Incubation for 7 days followed, when final readings were taken. Varied reactions have been obtained by making these double readings. Some organisms were found to be rapid acid formers, the reaction at the end of 7 days being no different from that taken at the end of the 24-hour period. The majority were slow acid producers, there being a negative reaction at 24 hours with a distinct positive reaction at the time of final observation. A third limited group produced a slight degree of acidity with certain of the sugars during the first 24 hours. Final readings at 7 days, however, showed a return to alkalinity.

The reactions to 8 sugars have been studied, namely, dextrose, levulose, and galactose of the monosaccharids, lactose, saccharose and maltose of the disaccharids, and mannite and dextrin among the polysaccharids. In the course of the work, the fermentive properties of about 502 strains have been determined. A number of duplicate strains were, of course, picked from the same plate during the examination of a given case, although an attempt was made to pick only colonies which showed apparent differences in growth characteristics. Likewise, certain cases have been studied over considerable lengths of time being frequently cultivated, and again similar strains were isolated from time to time. In the tabular summary of the fermentive reactions of the group, table 1, only case strains are included; that is, each strain



included in the table represents a particular variety of organism occurring in a given case and does not include duplicate strains isolated at the same time or in subsequent examinations. The reaction of 266 such case strains are presented. They represent organisms present in the nose and throat of normal persons and of persons suffering from influenza and from cases of common colds.

There are, on the basis of results presented in table 1, two main groups of gram-negative cocci present in cultures from the normal nose and throat, and in acute respiratory infection. One group of organisms includes those cocci which do not attack any of the carbo-

TABLE 1  
THE FERMENTATION REACTIONS OF GRAM-NEGATIVE COCCI OF THE NOSE AND THROAT

Organism	Number of Case Strains	Dextrose	Levulose	Maltose	Saccharose	Lactose	Galactose	Mannite	Dextrin
<i>M. catarrhalis</i> .....	98	—	—	—	—	—	—	—	—
<i>M. catarrhalis</i> Subgroup A *.....	5	± —	—	—	—	—	—	—	—
<i>Meningococcus</i> .....	8	+	—	+	—	—	—	—	—
<i>M. pharyngis siccus</i> .....	20	+	+	+	+	—	—	—	—
<i>Diplococcus crassus</i> .....	15	+	+	+	+	+	+	—	—
Chromogenic group 1.....	2	+	—	—	—	—	—	—	—
Chromogenic group 2.....	5	+	+	—	—	—	—	—	—
Chromogenic group 3.....	7	+	—	+	—	—	—	—	—
Chromogenic group 4.....	20	+	+	+	—	—	—	—	—
Chromogenic group 5.....	45	+	+	+	+	—	—	—	—
Chromogenic group 6.....	21	+	+	+	+	+	+	±	±

\* One strain fermented maltose as well as dextrose. Agglutination for meningococcus, negative.

Key: — = no fermentation; ± — = very slight acidity in 24 hours, alkaline 7 days; + = distinctly acid; ± = variable reaction on polysaccharides, but one or the other fermented or both.

hydrates, the *Micrococcus catarrhalis* group. The second general group is characterized by its chromogenic properties and may be divided into various subgroups on the basis of fermentation reactions. In addition to these two larger groups, meningococci have been isolated in a small percentage of cases. Other organisms encountered are *M. pharyngis siccus* and *Diplococcus crassus*. *Diplococcus mucosus*, observed by von Lingelsheim<sup>6</sup> in a study of gram-negative cocci in epidemic meningitis was not encountered. In addition to the organisms classified in the preceding table, a few strains did not fall into any of the recognized groups. Two chromogenic strains produced a rather well marked red pigment in contrast to the greenish-yellow pigment produced by other chromogenic gram-negative cocci. Sugar reactions showed acid production from dextrose, levulose, saccharose, lactose and maltose.

Three other strains produced an intensely yellow pigment. Of these 3 strains, 2 fermented dextrose and saccharose only, while the third fermented dextrose, levulose and saccharose. These 5 organisms are grouped together in succeeding tables as heterologous strains.

The largest single group of organisms is that group distinguished by lack of acid-producing power when grown on the carbohydrates tested. This group has been termed the *Micrococcus catarrhalis* group. It has been found to be representative of a group of organisms rather than a single distinctive species. While all of the 98 case strains included in the tabular summary have shown the common property of lack of fermentive power, morphologic and especially cultural studies indicate that there is a distinct difference among various strains. This fact has been noted by previous workers, notably by Elser and Huntoon<sup>7</sup> who distinguished 2 cultural types of *M. catarrhalis*. Based on strains studied in this work, the members of the group can be divided into 4 rather well marked subgroups, all of which fail to ferment carbohydrates, with possibly a fifth, designated in the table as *M. catarrhalis* subgroup A, which differs from the rest in its reactions toward sugars. The largest subgroup among the nonfermenting types corresponds to the classical description of *M. catarrhalis* given by Ghon and H. Pfeiffer.<sup>14</sup> The colonies appear after 24 hours as somewhat raised, opaque, white or gray-white disks, about the size of a meningococcus colony. When examined under the dissecting microscope, they are seen to have an irregular serrated margin and may be confused with colonies of certain diphtheroid organisms. The colony is typically granular, differing in this respect from the meningococcus colony which is more homogeneous. The different elements of the colony are strongly adherent to each other, and fragments may be detached with a needle. It differs in this toughness and tenacity of structure from most of the other types of gram-negative cocci. Morphologically the coccus is gram-negative and usually somewhat larger than the meningococcus. It characteristically appears in diplococcus form, although occasionally strains show numerous tetrads. Like most of the members of the group, it shows an early tendency to present degeneration forms, and even in young cultures, particularly in older ones, variations in size of different cells is readily observed. The cocci retain the Gram stain in some degree as swelling and degeneration progress. Irregularly stained preparations result, some cells being

<sup>14</sup> Ztschr. f. klin. Med., 1902, 44, p. 262.

gram-negative, others, especially the giant forms, appearing gram-positive. These organisms grow readily in subculture and usually can be maintained on starch agar for long periods of time without difficulty. Occasionally certain strains exhibit a tendency to die out for no apparent reason, even after a long period of cultivation on artificial medium. Daily subcultures and use of blood medium are necessary to restore satisfactory growth. Sixty-eight of the 98 case strains studied belong to this division.

The second subgroup corresponds in all respects to the preceding organism in colony structure, morphology and growth characteristics. It differs, however, in that a pale yellow pigment is formed by cultures grown on starch or plain veal-infusion agar. It was believed at first that these strains must belong to the group of chromogenic cocci so commonly found in the nose and throat, although the color of pigment formed was not exactly characteristic of the latter, which is a greenish yellow. Repeated transfers have been made to carbohydrate mediums in the course of the study of these strains, but never have they shown any indication of acid-producing power. Four such case strains have been isolated. One was obtained from a normal case, cultivated weekly for 3 months, with the coccus consistently present in all examinations.

A third subgroup, including 18 like strains, showed little cultural resemblance to the former 2. The colony differs markedly and indeed has much the appearance of a meningococcus colony, except that it is smaller. They are about one-half the size of the predominating type of *M. catarrhalis* colony and are flat, gray, rather than whitish gray, and translucent. Structurally, the colony is homogeneous, without granular appearance, as a rule, although slight granulation may appear toward the center. The surface is smooth and glistening while the edges are regular, in contrast to the jagged outline of the more common *M. catarrhalis* colony. In all respects it resembles a small, meningococcus colony. Morphologically the organism has practically the same appearance as other forms mentioned. Growth of recently isolated cultures is usually scant on primary isolation, but generally increases in amount after continued cultivation. Growth at best was irregular, however, and it was this group which gave the most trouble in maintaining stock cultures. Growth characteristics remained unchanged, however, with most of the strains, but 4 of them, after several months on artificial medium, gradually presented an appearance more nearly resembling the type of growth of the typical *M. catarrhalis*.

Another group of 8 strains differed a great deal from all other strains studied. The colonies were almost pinpoint in size, being extremely minute. They were transparent, glistening and possessed smoothly rounded edges. Such cultures, even when grown artificially for some time, have failed to show any change in characteristics, and maintain the same delicate growth. Cultures are initiated with great difficulty on other than blood agar, and to maintain them it has been necessary to transfer at weekly intervals, using blood medium. These strains are also distinct morphologically. Although derived from this fine delicate appearing colony, stained preparations of the coccus made after 24 hours' growth show the presence of a giant form of coccus with frequent metachromiasis. These stained preparations had all the appearance of a like preparation made from an old culture of the first type of *M. catarrhalis*, so much so that a series of stains were made from these cultures at early periods of growth, at 4, 6, 8 and 12 hour periods, to ascertain whether at these stages the organism might not present the usual morphology. At the 4-hour period the cocci were larger than the ordinary gram-negative coccus, but later at 8 and 12 hour periods the morphologic picture was much like that of the ordinary *M. catarrhalis*. Later the giant forms appeared. It would seem, then, that these strains represent a type of organism allied to the others, but undergoing more rapid transition into degenerative forms.

The last subdivision of the group consists of about 5 strains which agree exactly, in morphology and in growth characteristics, with the predominating type. They are different, in that they give rise to a slight degree of acid production on certain of the sugars—always dextrose, and maltose, as well in the case of one of the strains. The degree of acidity produced is slight, just sufficient to impart a delicate pink color to the medium. This acidity is transitory, however, and readings at 48 to 72 hours show a loss of color, with 7-day readings indicating a final alkaline reaction. It was felt that minute differences in the reaction of different lots of medium might account for the slight initial acidity, but repeated tests gave like results. Since observations have been based on 7-day readings, these strains have been classed as members of the *M. catarrhalis* group, and are indicated in the table as "*M. catarrhalis* subgroup A." It is quite probable that these strains belong to the same group, but represent a distinct variety.

The group of gram-negative cocci which are classed as *M. catarrhalis* can then be divided into 4 definite subgroups on the basis of cultural differences and to a certain extent on morphologic variation. An additional subgroup is recognized which is characterized by a slight initial acidity with certain carbohydrates, followed by a terminal alkalinity. All have the common property of spontaneous sedimentation when suspended in salt solution or nutrient broth, although that property is less marked in members of the third subgroup described.

A number of gram-negative cocci, possessing a common property of pigment production, but differing widely in fermentive properties, constitute a group second in incidence to the *M. catarrhalis* group. With the exception of chromogenic group 6, all of these organisms agree morphologically, and growth characteristics are for all practical purposes the same. Slight variations in the quality of the pigment are evident in different strains, but in general the colonies when viewed by transmitted light have a greenish yellow color and are semi-opaque. Viewed by reflected light, the color of colonies on agar appears to be greenish gray. They are somewhat smaller than colonies of the *M. catarrhalis* group, being about 1 mm. in diameter at 24 hours. Rather distinct variations in colony appearance are evident in different strains. Some of them closely resemble that of *M. catarrhalis*, while others, particularly strains belonging to chromogenic group 3, are quite like a meningococcus colony. The usual colony appearance, however, is that of an irregularly circular disk with more or less jagged outlines and a varying granular structure. Most of the colonies possess the dense, compact qualities peculiar to the *M. catarrhalis* colony and can be broken up into numerous crumbly fragments when touched with a needle. The organisms of chromogenic group 6 differ from the rest. The organism occurs usually in pairs or tetrads, but occasionally decided clumps are observed in stained preparations. Colonies appear more opaque than those of other chromogenic cocci and are larger. The pigment is usually a pale yellow and does not possess the greenish tint common to the rest.

On the basis of sugar variations, the chromogenic organisms can be divided into 6 different groups. Three small groups ferment, respectively, dextrose only, dextrose and levulose, and dextrose and maltose. A larger group ferments 3 of the carbohydrates—dextrose, levulose and maltose. The largest group, constituting about one-half of the strains having pigment producing power, ferments dextrose, levulose,

maltose and saccharose. Group 6 ferments uniformly all of the monosaccharids and disaccharids employed, and always one or the other of the 2 polysaccharids, sometimes both. It is probable that members of this group, judged by their morphology, growth characteristics and the quality of the pigment produced, are in the light of the fermentation reactions to be regarded as aberrant or degenerated forms of the staphylococci. They were, however, consistently gram-negative and are classed with the other chromogens. The probable relationship of certain other types of gram-negative chromogens to the staphylococcus, particularly organisms corresponding to the chromogenic group 5 of this classification, has been pointed out by von Lingelsheim.

*M. pharyngis siccus* has been encountered a number of times. The colonies of this organism are readily distinguished from other types of gram-negative cocci by their decided firmness of structure. It is almost impossible to break them up with a needle, and indeed the whole colony can be lifted bodily from the surface of the medium. They are closely adherent to the medium and about the size of the *M. catarrhalis* colony. Morphologically the cocci occur in pairs, but are distinctly smaller than other gram-negative cocci observed.

*Diplococcus crassus*, described in detail by von Lingelsheim,<sup>6</sup> occurred in 10 cases of the series. It retains the gram stain with more tenacity than other gram-negative cocci, and usually weakly gram-positive and gram-negative members can be observed in the same preparation, especially in older cultures. The colony is small, grayish white and rather compact.

Meningococci constitute the last division in the group. The morphology and characteristics of growth are too well known to require detailed description. It will be noted that chromogenic group 3 and the meningococci have identical sugar reactions. In all cases, confirmation of meningococci has been made by agglutination with a polyvalent meningococcus serum.

Analysis of this classification of gram-negative cocci by cultural methods establishes the fact that the *M. catarrhalis* group has the most common incidence in cases of common colds and influenza, and in normal throats. Chromogenic cocci occur frequently, but the incidence of any particular type is not marked. The chromogens have been studied serologically by Elser and Huntton<sup>7</sup> who demonstrate that organisms corresponding to our group 5, the most common chromogen in the upper respiratory tract, are immunologically different,

although agreeing in fermentation. Strains corresponding to the present groups 2 and 3 were serologically the same. Further study of these organisms has not been attempted on account of the conclusive results of these workers, and because no group occurs with any decided frequency in cases of respiratory infection. It has seemed important to determine whether or not the different strains of *M. catarrhalis*, which have been found to occur so frequently, are representative of a single dominant type in this large percentage of colds and influenza and are capable of being distinguished from types of the same organism occurring in the normal nose and throat. For this reason a serologic study of representative strains has been undertaken.

Agglutination tests could not be used for, unfortunately, all of the strains with the exception of 8 of the cultural type 3, and 1 strain of the cultural type 1, had the undesirable quality of spontaneous sedimentation. Attempts were made to obtain stable suspensions by various means, such as variation of the salt content of the medium in which they were taken up, and by the addition of a minute amount of alkali or acid to the suspension, but without success.

The technic of complement fixation seemed to furnish the most practical method. Before undertaking a serologic classification by this technic, preliminary tests were made to determine the extent to which antibodies for *M. catarrhalis* could be developed in an experimental animal. Rabbits were injected intravenously with typical strains. Immunization is relatively easy for the animals will tolerate large initial doses of live organisms. Bacteriolytic experiments with immune serums, plus complement and a standardized suspension of cocci demonstrated that strongly lytic serums could be prepared. Serum for one of the agglutinable strains gave an agglutinin titer of 1:2,000.

Antigens were prepared for 15 different strains representative of the various cultural types, and cross-fixation experiments, following standardization of the antigens with polyvalent serums, have been undertaken. The results are as yet incomplete and will be reported in a later paper. Sufficient data are at hand to determine that the various strains which are alike on the basis of suar reactions are not representative of a single type of organism when judged by complement-fixation reactions. At least 3 different types can be differentiated. Whether the serologic grouping can be correlated with the 4 cultural types which have been described, cannot yet be determined.

## THE GRAM-NEGATIVE COCCI IN THE NORMAL RESPIRATORY TRACT

The chief aim of this investigation has been directed toward a more exact knowledge of the relationship of these gram-negative coccal forms to acute infections of the upper respiratory tract. The work on classification was preliminary and essential to any interpretation of the significance of the organisms observed in various pathologic conditions. Definite knowledge of the incidence of the various groups of gram-negative cocci has been sought likewise in the normal nose and throat in order that a workable basis for comparison with the incidence under pathologic conditions might be available.

The normal carrier rate for the meningococcus has been the subject of extensive investigation during epidemic periods and depends largely on the extent of contact of the so-called normal population. In non-epidemic times the number of persons who carry meningococci in their upper respiratory tract varies, according to most authorities, from 3 to 6%, but in epidemic periods rises much higher. Frequent references in the literature to the presence of *M. catarrhalis* in disease conditions are available, dating from the original work of Ghon and Pfeiffer. No comprehensive study of the later organism in reference to its occurrence in the normal respiratory tract can be found. Arkwright<sup>15</sup> made nasal examinations of 15 normal subjects and found organisms which he classed as *M. catarrhalis* in 5 cases, or 33%. No information concerning the incidence of the other varieties of gram-negative cocci under normal conditions could be found. A series of 6 normal persons were studied by Bloomfield<sup>16</sup> at weekly intervals over a period of from 1 to 3 months. In practically all cultures of the series the predominating organisms were found to belong to the group of gram-negative cocci. No attempt was made to differentiate species other than the meningococcus. It seemed highly desirable to determine a normal incidence for all types, particularly a rate which would have been established by the same methods, and under the same conditions, as the subsequent study of respiratory conditions.

Various groups of normal persons have been studied at different times of the year in order to obtain an idea of the general incidence, as well as seasonal variations. One series was cultured during the spring months, April and May, a second during the summer months, June, July and Aug., while the third group was studied during Nov.,

<sup>15</sup> Jour. Hygiene, 1907, 7, p. 145.

<sup>16</sup> Bull. Johns Hopkins Hosp., 1921, 32, p. 33.



Dec., Jan., and Feb. The winter group includes cases from both 1919-20, and 1920-21. All of the subjects for study were drawn from the general student population at the university, and are probably representative of the ordinary urban community. No person in these groups had at the time of examination been knowingly exposed to colds, nor had they suffered from colds within recent date. A careful clinical examination was made before culturing to rule out possible inflammation of the respiratory mucous membrane. The results of the study of the nose and throat of normal persons are presented in table 2.

TABLE 2  
THE GRAM-NEGATIVE COCCI IN THE NORMAL NOSE AND THROAT

Series	Number Examined	M. Catarrhalis	M. Pharyngis Siccus	Diplococcus Crassus	Meningococcus	Chromogenic					
						Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Spring.....	35	15	1	0	0	0	0	0	1	8	4
Summer.....	47	24	7	2	1	0	0	1	5	6	2
Winter.....	28	12	2	2	0	0	1	0	4	4	2
Total.....	110	51	10	4	1	0	1	1	10	18	8
Percentage incidence all cases.....	..	46	9	4	1	0	1	1	9	17	7

This summary presents the total number of subjects included in each series and indicates likewise the number from which organisms of the various groups were isolated. The group which does not possess fermentive power, *M. catarrhalis*, constitutes by far the most common type observed in the respiratory tract. Various types of chromogenic cocci come next in order. *M. pharyngis siccus* is present in 9% of persons. The most significant fact brought out by these experiments, conducted as they were at different times of the year, is the practical absence of any seasonal variation. All of the organisms, including the most common group, *M. catarrhalis*, show practically the same percentage incidence at all times of the year. It was expected that the summer series might show a lower incidence than that observed during the colder months when respiratory infections are more prevalent. Such did not prove to be the case.

#### THE GRAM-NEGATIVE COCCI IN COMMON COLDS

*M. catarrhalis* is the only organism among the group of gram-negative cocci, to which any importance has been ascribed by previous

workers, as a possible factor in the cause of infections of the upper respiratory tract of the type of common colds.

The organism when originally observed by Seifert,<sup>17</sup> was found by him in the sputum and nasal secretions of persons affected by a small epidemic of infectious bronchitis. Later the organism was encountered by R. Pfeiffer<sup>18</sup> who obtained it in culture and gave it its name. He found it in large numbers in the bronchioles and alveoli in children with bronchopneumonia. Frosch and Kolle,<sup>19</sup> in subsequent studies, found it in other cases of bronchitis. The organism observed by Ritchie<sup>19</sup> in a case of bronchopneumonia in a child was doubtless the same organism. Numerous early investigators who give indeterminate descriptions of "gonococcus-like organisms" or "meningococcus-like organisms" as encountered in the simpler respiratory infections were probably dealing with *M. catarrhalis*. Ghon and H. Pfeiffer<sup>14</sup> made an extended study of the organism, which they found in numerous cases of acute bronchitis and bronchopneumonia. Lord<sup>20</sup> has observed *M. catarrhalis* in the sputum of about 12 patients with ordinary bronchitis. Arkwright<sup>15</sup> isolated this coccus from 11 of a series of 33 cases of catarrhal inflammation of the nose. Voorhies<sup>21</sup> and likewise Mackay<sup>22</sup> state that common colds are due to a number of organisms, among them *M. catarrhalis*. *M. catarrhalis* observed in most of the cases reported by these investigators has usually been associated with some other organism, such as the pneumococcus, the streptococcus, or the bacillus of Pfeiffer, although in a smaller number of cases, it has been found essentially as the only organism involved.

In the present study, investigation has not been confined to a determination of the prevalence of *M. catarrhalis* in acute upper respiratory infections, but an attempt has been made to determine the complete gram-negative flora. It seemed desirable to observe the degree of association of the other groups of gram-negative cocci with *M. catarrhalis* and with other organisms in respiratory infections, with the possibility in mind that they might exercise some symbiotic relationship, if no distinct pathogenesis. Our cases of colds have been obtained from the same general student population at the University of Chicago that furnished the subjects studied in the several normal series. No attempt has been made to select certain types of infection. All cases were examined as reported from time to time. Consequently the cases included in the "cold" series vary considerably in the clinical type of infection. A special effort was made to have cases available early in

<sup>17</sup> Samml. klin. Vortr., Leipzig, No. 240, 1882.

<sup>18</sup> Flügge, C.: Die Mikroorganismen, 1896.

<sup>19</sup> Jour. Path. & Bacteriol., 1900, 7, p. 1.

<sup>20</sup> Centralbl. f. Bakteriол., I, O., 1903, 34, p. 641.

<sup>21</sup> Amer. Med., 1917, 12, p. 125.

<sup>22</sup> Brit. Med. Jour., 1919, 2, p. 159.

the course of the inflammation. The majority were observed within the first 24 hours, rarely after a period of more than 48 hours following the onset of symptoms.

A total of 119 colds are included in the present series. A summary is presented in table 3 of the gram-negative cocci present, classified according to fermentation reactions, and subdivided according to differences in clinical diagnosis of the subject.

TABLE 3  
THE GRAM-NEGATIVE COCCI IN COMMON COLDS

Type of infection	Number Cases Examined	Micrococcus Catarrhalis	M. Catarrhalis Sub-group A	M. Pharyngis sicus	Diplococcus Crassus	Meningococcus	Chromogenic Group						Heterologous
							1	2	3	4	5	6	
Acute rhinitis..	55	25	1	5	4	3	1	2	2	4	12	4	2
Acute rhinitis and bronchitis..	9	5	0	1	0	0	0	0	1	1	4	0	0
Acute rhinitis and acute pharyngitis..	6	4	0	0	0	1	1	0	0	0	0	1	0
Acute rhinitis and tonsillitis	2	1	0	1	0	0	0	0	0	0	0	1	0
Acute rhinitis and sinusitis..	2	2	0	0	0	0	0	0	0	1	0	0	0
Acute pharyngitis.....	14	5	2	0	2	0	0	1	1	1	3	2	1
Acute pharyngitis and bronchitis.....	3	0	0	0	0	0	0	0	0	0	1	2	0
Acute tonsillitis.....	11	5	0	0	1	0	0	1	0	1	1	1	0
Acute tonsillitis and bronchitis.....	1	0	0	0	0	0	0	0	0	0	1	0	0
Epidemic tonsillitis and pharyngitis...	16	7	6	1	0	1	0	0	0	1	5	1	1
Acute laryngitis.....	1	0	1	0	1	0	0	0	0	0	0	1	0
Total colds..	119	54	4	8	7	5	2	4	4	9	27	13	4
Percentage incidence in all cases.....	...	45	3	7	6	4	2	3	3	8	23	11	3

One striking feature is evident when the results obtained from the study of the gram-negative cocci in colds are compared with the figures derived from the study of normal persons. The incidence of the various groups which have been differentiated agrees almost exactly in both instances. The frequency of *M. catarrhalis* in the normal and the pathologic throat is essentially identical, 46% in normal persons and 45% in persons with various types of colds. Similar agreement exists for most of the other groups.

No decided variation in the incidence of various groups can be observed in the different clinical forms of inflammation which have been studied. If one may compare those cases in which the infection was confined to the nose or pharynx with the fewer cases which presented bronchial involvement as well as inflammation of some portion of the respiratory tract higher up, it would seem that gram-negative cocci, particularly *M. catarrhalis*, occur less frequently in the latter type of cold.

In the course of the work, no attempt was made to determine the exact numbers of gram-negative cocci in given plate cultures in relation to members of the pneumococcus, streptococcus, staphylococcus and Pfeiffer bacillus groups. Rough plate readings were made, however. While colonies of the general gram-negative character could be observed in the majority of cultures—somewhat more than three-fourths of them—nevertheless it was uncommon to find the gram-negative flora constituting more than 10% to 30% of colonies. Occasionally plate cultures would be dominated by these organisms, sometimes constituting from 80% to 90% of all colonies.

#### THE GRAM-NEGATIVE COCCI IN INFLUENZA

A recurrent epidemic of influenza of short duration occurred during the early part of 1920, following the pandemic of 1918. The work which we were doing on common colds was suspended and our investigations were confined to a similar study of the incidence of gram-negative cocci in influenza. Only a relatively small number of cases could be investigated, due to the limited time that material was available. Three different groups of cases were studied, all occurring within the vicinity of Chicago. It was felt that if cases could be studied which developed in different localities where contact between groups was unlikely, that possibly the comparison afforded might permit a generalization on the bacteriologic conditions in this type of infection.

The presence of *M. catarrhalis* in influenzal conditions was demonstrated as early as 1890, when Kirchner<sup>23</sup> first reported it as being one of the organisms encountered in influenza, prevalent at that time in epidemic proportions. Subsequent to the epidemic of 1890, several instances are on record in which the organism has been found in inter-epidemic outbreaks of influenza-like infection. In certain instances the

<sup>23</sup> Ztschr. f. Hyg. u. Infektionskr., 1890, 9, p. 528.

organism has been observed in connection with certain others of the common respiratory micro-organisms, such as the pneumococcus or the streptococcus. At other times, it has been considered by various writers as the chief organism involved in particular outbreaks. The studies of Ghon and H. Pfeiffer<sup>14</sup> have been referred to. In 1905, Dunn and Gordon<sup>24</sup> record an epidemic simulating influenza in which *M. catarrhalis* seemed to be the predominating bacterium. Bezançon and De Jong<sup>25</sup> describe an epidemic occurring the same year in Paris, with *M. catarrhalis* dominant in bacterial cultures.

In the most recent pandemic of influenza, a great number of workers found *M. catarrhalis* in cases distributed throughout the country. Practically every bacteriologic study contains some reference

TABLE 4  
THE GRAM-NEGATIVE COCCI IN INFLUENZA

Case Series	Number Cases Examined	Micrococcus Catarrhalis	<i>M. catarrhalis</i> Sub-group A	<i>M. Pharyngis</i> siccus	Diplococcus Crassus	Meningococcus	Chromogenic Group						Heterogeneous
							1	2	3	4	5	6	
University of Chicago.....	8	2	0	0	1	0	0	0	1	1	1	0	0
Great Lakes....	20	8	1	2	2	1	0	0	1	0	2	3	0
Camp Grant...	12	4	0	0	1	1	0	0	0	0	1	0	0
Total cases..	40	14	1	2	4	2	0	0	2	1	4	3	0
Percentage incidence a 11 cases.....	...	35	2	5	10	5	0	0	5	3	10	8	0

to the organisms, although no detailed study of its relationship to influenza has been encountered. No attempt will be made to summarize the work of the past few years. The general deduction may be drawn that while *M. catarrhalis* is one of the more common species encountered in epidemic influenza, it rarely occupies a dominant position in the bacterial flora. Little importance has been accredited this organism as a factor in the pathogenesis of influenza. Finally, Clark and Murphy<sup>26</sup> have reported *M. catarrhalis* as the predominating organism in a recurrent epidemic occurring at the same time as the present group of cases.

The group of cases included in this report is derived from three sources. A number of the cases developed among the same general

<sup>24</sup> Brit. Med. Jour., 1905, 2, p. 421.

<sup>25</sup> Bull. et mém. Soc. méd. d. hôp., 1905, 22, p. 165.

<sup>26</sup> Clark, P. F., and Murphy, E. J.: Scientific Proc. Amer. Assn. Bacteriologists, Abstr. Bact., 1921, 5, p. 21.

student population at the University of Chicago, from which we had been drawing our material for the study of common colds. A group of cases occurring at the Great Lakes Naval Training Station, Waukegan, Illinois, constitutes our second series. The third series was obtained from soldiers stationed at Camp Grant, Rockford, Ill. The results of the examinations from these three series of influenza cases are contained in table 4.

The total number of cases that it was possible to examine during the brief course of the epidemic is too small to warrant definite conclusions. The significant feature is the drop in the general incidence of gram-negative cocci. All forms, including *M. catarrhalis*, occurred less frequently in influenza cases than in common colds. Curiously enough, the incidence of *M. catarrhalis* was found to be lower in cases of influenza than in the group of normal persons. It would seem that in these cases at least, the gram-negative forms had been crowded out or overgrown by some more virulent organism, judged by the normal incidence level established in previous studies.

#### AN ATTEMPT TO DETERMINE THE SPECIFIC RELATIONSHIP OF *MICROCOCCUS CATARRHALIS* TO COMMON COLDS

Experimental data have been presented which furnish evidence of the presence of *M. catarrhalis* in a large percentage of common colds and influenza. The same group of organisms, however, has been found in normal persons just as frequently. If any significance was to be attached to the presence of this organism in the pathologic nose and throat, it seemed necessary to investigate certain possibilities. It was necessary to determine, whether in given cases of colds, *M. catarrhalis* had been present on the mucous membrane as a normal inhabitant previous to the development of the infection. If it were present for some time before the onset of symptoms, did it increase perceptibly in numbers with the developing infection, and thereby exercise a possible symbiotic relationship with the real causative agent of the inflammation? There was also the possibility that certain colds might be encountered which would show no previous carrier condition of *M. catarrhalis*, but presence of the organism during the infection. In such cases, it was important to determine whether the organism gained entrance into the inflamed area coincidentally with developing symptoms, previous to that time, or somewhat later in the course of the infection. If these points could be settled, more exact data regarding the specific relationship of *M. catarrhalis* would be available.

With these ideas in mind, a group of 10 normal persons was selected, and each one subjected to routine daily cultures over a period of 2½ months. Each subject was watched closely for the onset of symptoms which would indicate an oncoming cold. Following the initial examination, detailed studies of each culture were not made, except when colds developed. The routine procedure consisted of a critical examination of the plates each day, when an estimated percentage was recorded of the number of different types of colonies which had developed. This included the 4 more important groups, the green-producing colonies, the Pfeiffer-like colonies, staphylococcus, and gram-negative-like colonies. From time to time, at intervals of perhaps 2 weeks, detailed studies were made of gram-negative-like colonies in order to maintain a general idea of the specific species comprising that flora.

During the course of these experiments, 5 of the 10 subjects in the series developed one or more colds, the other 5 remaining normal throughout the period of observation.

Gram-negative cocci were isolated from all of the subjects who remained normal, at some time during the course of the experiments. They were present in variable numbers in practically all plate cultures during the series. Only 2 of them, however, subjects A7 and A5, gave cultures of *M. catarrhalis* while under observation.

Subjects A1, A2, A3, A6 and A9 developed colds at different times during the experiments. Subject A1 showed gram-negative cocci of the *Diplococcus crassus* type when first examined, with *M. catarrhalis* absent. Gram-negative-like colonies were not numerous. An acute pharyngitis developed about 2 weeks later, at which time *M. catarrhalis* was identified and gram-negative-like colonies constituted 45% of total colony development from swabs. Following the cold, catarrhalis-like colonies dropped to about 10 or 15% of all colonies. A little more than a month after the first pharyngitis, a second inflammation and cough were observed. Again catarrhalis-like colonies increased in relative numbers, beginning noticeably the day before symptoms appeared, when they constituted 50% of all colonies. On the day that the cold was apparent, 30% of gram-negative-like colonies were present, the next day 40%, and the second day, 80%. *M. catarrhalis* was demonstrated at this time and likewise 4 days later. Following the cold, the incidence of gram-negative-like colonies fell to a level of from 10 to 20%, with *M. catarrhalis* unable to be demonstrated.

The initial examination of subject A2 showed a considerable percentage incidence of gram-negative-like colonies. The organisms were found to belong to chromogenic group 5. On the second day after initiation of study, an acute rhinitis developed. *M. catarrhalis* could not be demonstrated. Three weeks after the first cold, a second rhinitis was diagnosed. Gram-negative-like colonies were few in number, almost negligible, and these few were the same chromogen 5. On the third day of the infection gram-negative-like colonies had increased in number to 30% of the total number of colonies, and *M. catarrhalis* was isolated, together with the chromogen. They continued to increase in numbers, and the next day the group represented 50% of the flora.

The history of subject A3 resembles that of A2 as regards incidence of colds. An acute rhinitis developed the second day of observation. Large numbers, 50%, of chromogen 5 organisms were observed early in the infection, and indeed colonies of the gram-negative type dominated the plates. *M. catarrhalis* was not isolated until 8 days after the beginning of symptoms, and its presence was transitory as it was negative 2 days later and continued to remain so. A second rhinitis developed about 6 weeks later, and although gram-negative-like colonies showed an increase in numbers, *M. catarrhalis* could not be found.

Subject A6, at the beginning of the study, had a low incidence of gram-negative-like colonies which were of the chromogenic groups. An acute rhinitis developed in 3 weeks, but no marked increase in gram-negative organisms could be observed, and *M. catarrhalis* was absent. *M. catarrhalis* was only isolated once during the study of cultures from this subject. Almost a month after the termination of the cold, there was a sudden flare-up of catarrhalis-like colonies with a colony percentage incidence of 85%. *M. catarrhalis* was identified. This marked increase was transitory, the number decreasing shortly with *M. catarrhalis* absent in cultures thereafter.

The gram-negative cocci were apparently of little importance in the history of subject A9. They could be identified in most cultures as constituting about 10% of the flora. *M. pharyngis siccus* and group 5 chromogens were identified. *M. catarrhalis* was isolated at no time, and although a mild pharyngitis developed 3 weeks after the start of the study, no relative change could be observed in the gram-negative flora.

Analysis of the 5 cases in which colds developed brings out certain facts. A number of colds are encountered with which *M. catarrhalis*



is concerned in no way. Although gram-negative cocci could regularly be isolated during the extended examinations from subjects A3, A6 and A9, they apparently were concerned in no way with the colds which developed. *M. catarrhalis* was demonstrated in both subjects A3 and A6 but could not logically be connected in any way with the colds which were observed. A particular point of interest observed in subject A6 was the sudden and marked increase in gram-negative forms which occurred without apparent effect on the mucous membrane. *M. catarrhalis* constituted 85% of all colonies present on plates at that examination. Such transitory domination of the flora by a particular organism seems to demonstrate the frequently changing character of the bacterial flora in at least a considerable proportion of persons. Seemingly, a sudden dominant position in the flora can be attained by several organisms, possibly through invasion of the respiratory mucous membrane by a strain more virulent than its fellows. Such instances have been observed for the pneumococcus, *Bacillus mucosus capsulatus* and the Pfeiffer bacillus as well as *M. catarrhalis*, in a study of normal throats over extended periods.

The two cases of rhinitis which occurred in the study of subject A1 influenced certain changes in the gram-negative flora. *M. catarrhalis* had not been present in cultures previous to the infection. Increased numbers of gram-negative-like colonies were observed the day before symptoms and during the height of the infection. Gram-negative forms, including *M. catarrhalis*, were distinctly dominant in plate cultures. With subsidence of the infection the organisms tended to disappear. The tremendous increase in numbers may have been due to the acute process so altering the environment that the organisms took hold and grew more rapidly at the seat of the disease. In the rhinitis of subject A2, *M. catarrhalis*, which was observed late in the cold, can probably best be interpreted as one of the secondary invaders.

Eight colds, then, have developed in these 5 persons. *M. catarrhalis* was apparently concerned in no way with 5 of them. In one, it seems to have invaded secondarily the previously inflamed area. Two cases may have been caused by *M. catarrhalis*. It has been emphasized in a previous paper, however, that one cannot ascribe a causative relationship to a given organism, even when the evidence is reasonably favorable, for there is no direct knowledge of the period of incubation of such infections. The presence of *M. catarrhalis* at the time of infection may have meant merely that under the stimulus of the true exciting

agent of the cold, these organisms, normally in the throat, although present in such small numbers that they could not be demonstrated, rapidly began to multiply, and possibly acquired as well a distinct pathogenicity. Thus one cannot determine whether the presence of the organism indicated a direct or merely symbiotic relationship to the infection.

THE EXTENT OF THE CARRIER STATE FOR MICROCOCCUS  
CATARRHALIS

Organisms of the type of *M. catarrhalis* have been demonstrated in essentially the same relative number of throats, whether they be normal or pathologic. Such being the case, interest is attached to the question of whether the organism is to be considered as a permanent inhabitant of the throat, with such frequent occurrence, or whether the presence of the organism in a given throat is limited, and its status subject to frequent change.

*M. catarrhalis* does not appear to be carried in appreciable numbers in throats of convalescents from colds. No general deduction can be made from the few cold cases in the "A" series, in which *M. catarrhalis* was involved. It was found in these cases, however, that *M. catarrhalis* was carried a relatively short time following convalescence.

More important, particularly in view of the common incidence of *M. catarrhalis*, is the relation of the organism to the normal person. Is the organism present for a long period of time in a given throat; is this the usual occurrence in a considerable proportion of normal people; and is it therefore most logically to be considered as a normal throat saprophyte? Or, on the contrary, does it inhabit a given throat for brief periods of time with the probability of frequent transfer from person to person?

Three different groups of normal persons have been studied at different times of the year. Cultures from the individuals of each group were taken at weekly intervals over a period of 2 months, from one group for 2½ months. The first group of 5 persons was studied during the winter months of Nov., Dec. and Jan. No complicating colds developed during the period of observation. Three of the 5 subjects did not show *M. catarrhalis*. In fact gram-negative-like colonies were in the minority in practically all of the plates. The types of organisms of the gram-negative group which were represented were members of the chromogenic group, with one case showing *M. pharyngis siccus*.

The fourth subject of the group possessed a throat flora which was consistently dominated by members of the gram-negative group. The percentage of colonies on the various plates, which were representative of gram-negative cocci, varied from 40 to 90%, and the usual finding approached the higher figure. *M. catarrhalis* was the chief organism among the gram-negative-like colonies, but chromogenic cocci were also isolated.

The flora of the last member of the group, subject 5, was likewise characterized by preponderance of gram-negative-like colonies over other forms. This condition continued with periodic variations throughout the period of observation. The flora consisted of *M. catarrhalis* as well as a chromogenic coccus of group 5.

A different group of 6 persons was studied in the same way during the Spring months, April, May and early June. Two of them were negative for *M. catarrhalis* during the experiments, although the familiar chromogens were observed in one. Three subjects, NC 56, NC 154 and NC 505, gave positive cultures of *M. catarrhalis* throughout the period of observation. The other subject of the series, NC 228, harbored *M. catarrhalis* in the upper respiratory tract at the initial examination and for one month thereafter, following which cultures proved to be negative.

The third study of normal persons was conducted during the summer months, June, July and Aug. Five persons constituted this group. In cultures from 2 of them, S2 and S5, *M. catarrhalis* could not be found at any time. Chromogens were present in one case, *M. pharyngis siccus* in the other, while the latter likewise gave positive cultures of the meningococcus for 3 successive weeks, after which that organism disappeared. The other 3 subjects, S1, S3 and S4 all showed consistently cultures of *M. catarrhalis*. Subjects S3 and S4 gave positive cultures throughout the period of observation. Cultures from subject S1 failed to reveal the presence of *M. catarrhalis* at the initial examination but were positive thereafter until the work was terminated.

These separate groups of persons, studied during different seasons of the year, have given results which furnish rather conclusive evidence of the rôle played by the group of gram-negative cocci, and particularly by *M. catarrhalis*. The case incidence of the *catarrhalis* group, considering the various series of persons as a unit, corresponds to the same general percentage observed in the larger series of normal persons on whom a single examination was made. All subjects who

showed *M. catarrhalis* in throat cultures were, with a single exception, carriers of that organism throughout the various periods of observation. The one exception was positive at the beginning of the study but became negative for *M. catarrhalis* after one month. The previous length of the carrier state was of course indeterminate. One feels that this group of organisms, *M. catarrhalis*, constitutes a species which has become highly acclimatized to the mucous membrane of the upper respiratory tract. A large proportion of persons carry it in the throat for long periods of time without visible effect on the lining membrane. Rare instances may arise in which a more virulent strain may be involved in respiratory infection. In general it would seem to lead a saprophytic existence in numerous throats, a condition comparable to that of the commonly recognized saprophytes of the mouth.

COMPARATIVE VIRULENCE OF STRAINS OF *MICROCOCCUS CATARRHALIS*  
FROM NORMAL SOURCES AND FROM COLDS

*M. catarrhalis* has been demonstrated to be present in normal persons and in cases of colds with about equal frequency. In persons during influenzal infections it has been found less frequently than in normal persons. If any pathogenic properties are to be conceded the organism, it would seem essential to establish, among other things, a relatively heightened virulence of the coccus, when found in cases of colds and influenza.

Various strains have been tested in regard to their virulence for mice. These strains have been derived from different types of respiratory infection and from normal persons. While no definite information was at hand, it has been assumed that *M. catarrhalis* possesses the characteristic, common to so many bacteria, of a decline in virulence following continued cultivation on artificial medium. The following uniform procedure has been followed, in order that virulence might be tested as soon as possible after recovery of the strain from the throat, and likewise that directly comparable data might be obtained. Colonies were picked from blood-agar plates to blood-agar slants. Purity of culture was established by morphologic examination. Transfers were then made to dextrose medium, and if fermentation was not produced in 24 hours, a transfer was made from the original blood-agar slant to a second tube of the same medium. This 24-hour old culture was employed for the virulence tests. It was consequently in

its third generation on blood agar. By this method conditions were exactly comparable in all instances. Classification of the organism was verified later by the usual methods.

In preparing cultures for inoculation, care was taken to seed as nearly as possible the same extent of blood-agar medium. Growths were washed off in 2 c c of warm sterile broth and mice were inoculated intraperitoneally at once. In order to obtain an idea of the usual lethal dose for mice, preliminary tests were made with 4 strains from cases of colds. Mice were inoculated, respectively, with one-eighth, one-fourth, and one-half of the slant culture of the first strain. None of the mice died within 6 days. The next 3 cold strains were tested by inoculating one-eighth, one-fourth, one-half and one slant into a series of mice. Death occurred in 24 hours in all 3 animals receiving the massive doses of 1 whole slant. One animal injected with one-half slant died in 3 days, strain C557J. All others survived. It was felt that the use of such tremendous doses as one full slant, with an attendant volume of 2 c c did not constitute a test of any value when inoculated into so small an animal as the white mouse. In subsequent determinations, each strain was injected in doses of one-fourth and one-half slant. Eight normal strains have been tested and 8 cold strains. Death was produced by 2 of the normal strains, S3D and 505A2, in one-half agar slant doses. All other animals survived. Three of the cold strains produced death, C629D influenza, C557J rhinitis and bronchitis, and C82H16 rhinitis, at periods of 4 days, 3 days, and 2 days, respectively. The lethal dose of strains C629D and C557J was one-half slant, of strain C82H16 one-fourth slant. The larger dose of this strain, curiously enough, did not kill the animal. Profound symptoms of toxemia were evidenced in all of the animals receiving the larger dose and usually in those receiving the smaller. Necropsy cultures from the mice which succumbed failed to reveal the organism in the heart blood. Three cases C557J, C82H16 and S5D, gave cultures from the peritoneal fluid, C557J from the pleural fluid as well. The only pathologic changes which could be observed were a variable increase in the amount of peritoneal fluid, enlargement and hyperemia of the spleen, occasional subserous hemorrhage in the intestines and in one case increased pleural fluid. There were no indications of a generalized infection, pathologically or bacteriologically.

The organism possesses little or no virulence for mice. Tremendous doses were required to produce death which was evidently due

more to the toxic effect of the bacterial protein than any growth and reproduction of the organism in the tissues. Furthermore, no difference could be determined between the lethal effects of strains isolated from normal and from cold sources.

Various strains studied were practically avirulent for rabbits. No definite experiments were conducted to determine the degree of virulence, but in immunizing animals against numerous strains, doses of one-fourth blood-agar slant of live organisms were commonly employed, intravenously as the initial dose, followed by one-half and one whole slant on the next two days, without ill effect.

#### DISCUSSION

Cocci which fail to retain the Gram stain constitute one of the more common groups of bacteria which may be found in the upper respiratory tract of man in both health and disease. They can be divided on the basis of fermentative power and growth characteristics, into several different groups. The largest group encountered in this study is made up of those organisms which fail to produce acid through action on any of the more common carbohydrates. These organisms have been recognized as constituting a single group, *M. catarrhalis* which, however, may be divided into 5 subgroups on the basis of cultural differences. A second group of organisms, characterized by the production of a greenish yellow pigment, occurs in the human respiratory tract with about the same frequency as the first group. These strains likewise may be divided into 6 different subgroups on the basis of differences in fermentation. The meningococcus of Weichselbaum, *Diplococcus crassus* Kral, and *M. pharyngis siccus* complete the list of gram-negative cocci found.

The essential purpose of this study was to investigate the relationship which these gram-negative cocci bear toward upper respiratory tract infection. Certain data have been obtained. Some member of the general group can be found in three-fourths or more of all cases, *M. catarrhalis* in about half that number. The percentage of incidence of all groups is, however, essentially the same in normal as in pathologic throats. In fact, they are less frequent in persons with influenza than in normal persons, an indication that they have been crowded out by a more virulent species. No single group of gram-negative cocci, other than *M. catarrhalis*, occurs with special frequency in common

colds and influenza. The variable and irregular occurrence of these other groups would seem to limit any importance possessed by gram-negative cocci to the rôle played by *M. catarrhalis*.

Extended study of certain normal persons found to harbor *M. catarrhalis* in the throat has led to the conclusion that the organism is decidedly a permanent inhabitant of the throat, rather than a temporary invader. It lodges in a considerable percentage of throats, becomes readily acclimatized to that environment, and lives there for long periods of time, leading apparently a saprophytic existence. Finally, various strains from normal sources and from colds have been found to be avirulent for white mice and rabbits. Lethal effects have been obtained with a few strains, but only when massive doses were employed. Strains from normal persons and from cases of common colds present, moreover, no distinguishable differences in lethal power.

Rarely, circumstances occur in which it may be possible to place some emphasis on *M. catarrhalis* in explaining the pathogenesis of a given cold. Such an instance has been discussed in connection with the two rhinites observed in subject 1 of the "A" series.

Observation of *M. catarrhalis* in normal throats and in various respiratory infections, justifies but one conclusion. The organism is only rarely involved in the pathogenesis of acute infections of the upper respiratory tract in man. Based on its like incidence in colds and in normal persons, the actual decline in incidence in influenza, its long contained existence on the mucous membranes of many normal persons, and finally its lack of virulence, irrespective of origin from normal or pathologic throats, the logical deduction is that its usual sphere is that of a harmless saprophyte.

#### CONCLUSIONS

Gram-negative cocci which occur in the nose and throat normally and in acute upper respiratory infections may be grouped according to cultural characteristics and fermentative differences. Such a classification is presented in table 1 and the succeeding discussion.

No essential difference was distinguished between the incidence of the various groups of gram-negative cocci in common colds, and in a like series of normal persons. In epidemic influenza, the incidence was less than in normal persons.

*M. catarrhalis*, the most common member of the group, is carried for long periods of time in the throats of many normal persons, constituting a permanent member of the normal throat flora.

No distinguishable differences in virulence for mice and rabbits could be determined between strains of *M. catarrhalis* from normal sources and those from colds or influenza.

These observations do not indicate that *Micrococcus catarrhalis* is generally concerned in the pathogenesis of common colds or influenza.



# STUDIES ON THE CHEMOTHERAPY OF THE EXPERIMENTAL TYPHOID CARRIER CONDITION

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Typhoid fever has long been recognized as a disease which tends to produce the carrier condition. The carrier state may then persist for an unknown time which may be lengthy as noted by Gregg,<sup>1</sup> who claimed that in one case it has extended through fifty-two years, or it may be much shorter, clearing up within a few months. The percentage of carriers following cases of the disease seems to vary since Stokes and Clark<sup>2</sup> reported 1.85% in the course of a survey of 810 persons, while Semple and Greig<sup>3</sup> detected 11.6% after 86 individual examinations. It seems likely that the general mean ultimately will be found to lie between these two figures. The menace of such carriers to society becomes evident at once, and the impression becomes emphasized when we find that G. Mayer,<sup>4</sup> after careful work attendant on a survey of 405 cases, came to the conclusion that 32.3% of the new cases observed sprang from infection derived from carriers.

Typhoid is at first a generalized infection which later may become localized in one or more places in the body, and these remain for long periods foci of dissemination to the outside world. The principal one of these foci is the gallbladder. Here the disease is most resistant to attempts to clear it up. Many trials to sterilize this locus of infection have been made, and none has been admittedly successful. Chloroform has been advocated more or less enthusiastically by Conradi,<sup>5</sup> by Hailer and Rimpau,<sup>6</sup> the same two authors together with Ungermann<sup>7</sup> and by Bully<sup>8</sup> but Perussia<sup>9</sup> obtained negative results when working with artificially infected guinea-pigs. Liefmann<sup>10</sup> advocated the use of milk. Hertz<sup>11</sup> injected milk intramuscularly with results deemed to be favorable. Cinnamon oil alone out of an extensive series gave promising indications according to Hailer and Wolf.<sup>12</sup> Attempts to use iodine have been

Received for publication June 13, 1921.

<sup>1</sup> Boston Med. & Surg. Jour., 1908, 154, p. 80.

<sup>2</sup> Lancet, 1916, 190, p. 566.

<sup>3</sup> Government of India Bull., Calcutta, No. 32, 1908.

<sup>4</sup> Med. Klin., 1916, 12, p. 13.

<sup>5</sup> Ztschr. f. Immunitätsforsch., 1910, 7, p. 158.

<sup>6</sup> Arb. a. d. k. Gsndtsamte, 1911, 36, p. 409.

<sup>7</sup> Centralbl. f. Bakteriöl., 1911, 1, Ref., Beiheft, p. 112.

<sup>8</sup> Ztschr. f. Hyg. u. Infektionskr., 1911, 69, p. 29.

<sup>9</sup> Pathologica, 1912, 4, p. 141.

<sup>10</sup> München. med. Wchnschr., 1909, 56, p. 509.

<sup>11</sup> Wein. klin. Wchnschr., 1916, 29, p. 1920.

<sup>12</sup> Arb. a. d. k. Gsndtsamte, 1915, 48, p. 80.

made by Tsuzuki and Ishida<sup>13</sup> and by Kalberlah.<sup>14</sup> Indol was considered with favor by Roček.<sup>15</sup> Cummins, Fawcus and Kennedy<sup>16</sup> tried roentgen rays. The use of vaccines, while well established in prophylaxis, is not recognized as effective in treatment of the typhoid carrier although frequently used. Irwin and Houston<sup>17</sup> and Preti<sup>18</sup> have been the proponents, while Nichols<sup>11</sup> has opposed their use. Nichols also has suggested alkaline therapy as possibly worthy of consideration, but it is significant that in a later publication,<sup>20</sup> he has ignored entirely any such method. Finally, surgical interference has been resorted to by Dehler,<sup>21</sup> Leary,<sup>22</sup> by Nichols, Simmons and Stimmer<sup>23</sup> and by Henes.<sup>24</sup> Locle<sup>25</sup> reports the failure of cholecystectomy.

The multiplicity of expedients resorted to for the treatment of typhoid carriers, together with the fact that no one of them has received anything like general recognition, indicates that no satisfactory procedure thus far has been discovered. Further consideration of the possibilities of chemotherapy, particularly of dye therapy, seems desirable. The recent work of Gay and Morrison<sup>26</sup> on dye therapy in localized streptococcus infection would seem to offer little encouragement even in accessible and well localized infections. It must be admitted that success in reaching an infected gallbladder would seem even less likely than in the syndrome studied by these authors.

The qualifications of any compound in order that it may be administered successfully for chemotherapeutic purposes have been admirably and succinctly summarized by Jacobs.<sup>27</sup> It must be comparatively nontoxic to the tissues of the host, although it must have a selective affinity for the protoplasm of the invading organism. In addition, if it is to be effective for treatment of a gallbladder infection, it must be excreted in part at least through the bile and also must retain its germicidal power in bile.

It has long been recognized that many of the dye-stuffs, particularly those of the triphenylmethane and of the acridine series, are active in vitro against members of the colon-typhoid group. Certain of the stains, notably acridine compounds, are slow in velocity but ultimately highly efficient. Likewise, they have been demonstrated to show a considerable degree of specificity.

The initial stimulus for interest in dye-stuffs as germicides appears to have come from Roszahegyi,<sup>28</sup> who learned that methylene blue, gentian violet, vesuvin, fuchsin and methyl violet are not only bactericidal but also that they are selective within marked limitations. Bechhold and Ehrlich<sup>29</sup> confirmed Roszahegyi with regards to specific action, as likewise did Browning, Cohen and

<sup>13</sup> Deutsch. med. Wchnschr., 1910, 36, p. 1005.

<sup>14</sup> Med. Kln., 1915, 11, p. 581.

<sup>15</sup> Centralbl. f. Bakteriöl., I. O., 1915, 77, p. 100.

<sup>16</sup> Jour. Roy. Army Med. Corps, 1910, 14, p. 351.

<sup>17</sup> Lancet, 1909, 176, p. 311.

<sup>18</sup> Riforma med., 1917, 33, No. 41.

<sup>19</sup> Jour. Exper. Med., 1914, 20, p. 573.

<sup>20</sup> Ibid., 1916, 24, p. 495.

<sup>21</sup> München. med. Wchnschr., 1907, 54, p. 779 and 2134.

<sup>22</sup> Jour. Am. Med. Assn., 1913, 60, p. 1293.

<sup>23</sup> Ibid., 1919, 73, p. 680.

<sup>24</sup> Ibid., 1920, 75, p. 1771.

<sup>25</sup> Deutsch. med. Wchnschr., 1909, 35, p. 1429.

<sup>26</sup> Jour. Infect. Dis., 1921, 28, p. 1.

<sup>27</sup> Jour. Exper. Med., 1916, 23, p. 563.

<sup>28</sup> Centralbl. f. Bakteriöl., 1887, 2, p. 418.

<sup>29</sup> Ztschr. f. phys. Chem., 1906, 47, p. 173.

Gulbrandsen.<sup>30</sup> The work of Churchman<sup>31</sup> with relation to the action of gentian violet on gram-positive organisms aroused much interest in the United States. Kligler<sup>32</sup> states that gram-positive organisms generally are more susceptible to dyes. Dye-stuffs may have a much higher efficiency than phenol according to Kriegler,<sup>33</sup> and this is now a generally accepted fact. The toxicity of dyes is dependent on molecular structure and Kligler,<sup>32</sup> arrives at the conclusion that germicidal action is a function of the benzene nucleus. Likewise, he believes that enlargement of the number of alkyl radicles is attended by increase in toxicity. The close parallelism between degree of basicity and bactericidal power has been remarked also by Dreyer, Kriegler and Walker,<sup>34</sup> by Browning and Gilmour,<sup>35</sup> by Simon and Wood,<sup>36</sup> Graham-Smith,<sup>37</sup> Traube<sup>38</sup> and by Lewis.<sup>39</sup> Toxicity is augmented by the addition of side chains according to Browning and Gilmour,<sup>35</sup> Kligler<sup>32</sup> and Jacobs, Heidelberger and Amoss.<sup>40</sup> Simon and Wood<sup>36</sup> and Kligler<sup>32</sup> also state that if the side chain be ethyl rather than methyl, germicidal powers are increased to a still greater degree. The presence of a halogen in the molecule enhances its activity, according to Bechhold and Ehrlich,<sup>29</sup> and this statement apparently is confirmed by Jacobs, Heidelberger and Amoss.<sup>40</sup> Lewis<sup>39</sup> and also Simon and Wood<sup>36</sup> are authorities for the statement that the triphenylmethane series contains some of the most potent germicides.

It is a fact generally accepted that the presence of serum is incompatible with the bactericidal action of dye-stuffs, as it is with nearly all other bactericides. Bechhold and Ehrlich<sup>29</sup> encountered this difficulty. However, certain of the salts built up and tested by Jacobs, Heidelberger and Bull<sup>41</sup> did not show this drawback and Browning<sup>42</sup> and his co-workers are emphatic in their claims that flavine compounds show greatly improved effects with serum in contact. With these agree more conservatively Drummond and McKee<sup>43</sup> although Morgan,<sup>44</sup> Mueller<sup>45</sup> and Graham-Smith<sup>37</sup> do not come to like conclusions.

This survey of the possibilities of dye-stuffs indicates that it is conceivable that there may be anilin derivatives toxic to *B. typhosus* in vivo. In addition, since it is known that certain of them are excreted through the bile, it was felt that some of them might be found to sterilize infected gallbladders.

The stains selected for preliminary trial listed according to their classes in Green<sup>46</sup> together with the sources from which they were obtained were as follows:

<sup>30</sup> Jour. Path. & Bacteriol., 1919, 23, p. 124.

<sup>31</sup> Jour. Exper. Med., 1912, 15, p. 221; 17, p. 373; Jour. Am. Med. Assn., 1918, 70, p. 1047; 1919, 72, p. 1280; 1920, 73, p. 145.

<sup>32</sup> Jour. Exper. Med., 1918, 27, p. 463.

<sup>33</sup> Centralbl. f. Bakteriol., I. O., 1911, 59, p. 481.

<sup>34</sup> Jour. Path. & Bacteriol., 1911, 15, p. 133.

<sup>35</sup> Ibid., 1914, 18, p. 144.

<sup>36</sup> Am. Jour. Med. Sc., 1914, 147, pp. 247 and 524.

<sup>37</sup> Jour. Hyg., 1919, 18, p. 1.

<sup>38</sup> Biochem. Ztschr., 1912, 43, p. 496.

<sup>39</sup> Bull. Johns Hopkins Hosp., 1917, 28, p. 120.

<sup>40</sup> Jour. Exper. Med., 1916, 23, p. 569.

<sup>41</sup> Ibid., p. 577.

<sup>42</sup> Proc. Roy. Soc., 1918, 90, p. 136; Brit. Med. Jour., 1917, 1, p. 73; also footnote 35.

<sup>43</sup> Lancet, 1917, 193, p. 640.

<sup>44</sup> Lancet, 1918, 194, p. 256.

<sup>45</sup> Jour. Path. & Bacteriol., 1919, 22, p. 308.

<sup>46</sup> A Systematic Survey of the Organic Coloring Matters, 1914.

*Triphenylmethane*.—Basic fuchsin, Grübler; crystal violet, Grübler; new fast green 3B, Soc. Chem. Ind. Basle; saüregrün, M. L. B.; malachite green, Grübler; brilliant green, Grübler; erioglaucin A, Geigy; cyanin B, M. L. B.; setocyanin, Geigy; corallin (water soluble), Grübler; Spiller's purple, Grübler.

*Diphenylmethane*.—Auramine (Pyoktannin aureum), Merck.

*Thiazine*.—Methylene blue, Leitz.

*Disazo*.—Oxamine violet, B. A. S. F.; benzoazurin, Ber. Anil. Works; trypan blue, Cassella; Congo red, Grübler.

*Azine*.—Safranin, Harmer; neutral red, Grübler.

*Xanthene*.—Pyronine G, Eimer and Amend; rose bengal, Jager (Dusseldorf).

*Acridine*.—Proflavine, Boots Pure Drug Co., Ltd.; acriflavine, Lane Hospital, S. F.

All of these stains, with the possible exception of Spiller's purple, are used in many laboratories. These dye-stuffs were made up in a 1% solution in distilled water, and after a day they were streaked out on agar plates to determine their sterility. No difficulty with contaminating organisms was found.

Any dye-stuff to be active against *B. typhosus* in the carrier must retain its germicidal powers when mixed with bile. Likewise, the stain must pass through the circulation with its ability to kill the micro-organisms little or not at all impaired. With these limitations in mind, it was decided to make preliminary tests in vitro to determine what activity, if any, might be expected from the dye materials mentioned in the foregoing when in serum and in bile. Such stains as are found with germicidal activity in bile and in serum in glass may then be tested further in infected animals, provided they are not too toxic.

#### TECHNIC OF BACTERICIDAL TESTS

Tests then were carried out with one dye simultaneously in (1) horse serum which had been inactivated at 56 C. for one hour to destroy bacteriolytic and agglutinating power, (2) ox bile sterilized while still warm from the animal by autoclaving for 20 minutes at 15 pounds pressure, (3) beef broth and (4) physiologic salt solution. Salt solution was used in order that there might be present the standard in the series which has most often been resorted to by other workers and therefore in order to afford opportunity for comparison. As the hydrogen-ion concentration of the physiologic salt rose from  $P_H$  7.2 to approximately 5.0 during sterilization, due in all probability to the absence of buffer and changes in silicic acid from glass and in carbon dioxid, it became necessary to adjust the reaction to  $P_H$  7.2 after heating.

Small agglutination tubes cleansed with acid cleaning mixture, carefully rinsed with distilled water, plugged with cotton and sterilized in

the oven were the containers. In these, using sterilized pipets for measurement and transfer, the medium was placed to the amount of 0.9 c.c. Dilutions were made by adding to the first tube of each series 0.1 c.c. of 1% aqueous solutions of the dye. In case of compounds only slightly soluble in cold water but easily so in hot, it is necessary to have the solution and the pipet well warmed. Following careful mixing by means of the pipet, 0.1 c.c. of this was added to the second tube. Such mixing and transfer was continued until the series of dilutions was 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000. The last tube of the line was left without stain in order that it might serve as a check. Inoculations were made generally by the addition of two loopfuls of a 20-24 hour culture of *B. typhosus* to each tube and with the oese 2 mm. in diameter and of 0.4 mm. platinum wire.

Because it is recognized that the amount of liquid which may be transferred by one loopful varies greatly, effort was made to insert the needle always to the same depth, at the same angle with the meniscus and with the tube tipped to the same degree. Temperatures were always close to 37 C. The strain of *B. typhosus* was that which is recorded as "No. 3" in the records of this department. It was isolated originally from a carrier and used in other experimental work (Gay and Clappole<sup>47</sup>).

To determine possible germicidal power of the stains in the liquid mediums used, streaks were made by means of the same loop on agar plates immediately after the experiment was set up and the tubes inoculated. This series again was streaked out after 1, 5 and 24 hours. All prepared tubes and plates were kept during the intervals in the incubator at 37 C. Petri dish results were observed after 24 hours' incubation in reverse position and the results tabulated.

The results of these experiments are herewith presented. In order to save space the effects produced after 24 hours' exposure are the only ones given. Results are bactericidal rather than bacteriostatic.

The following stains are ineffective at a dilution of 1:1,000 in any one of the 4 mediums used; benzoazurin, congo red, corallin, cyanin B, eriochlorin A, neutral red, oxamine violet, sauregrün, setocyanin and trypan blue. Additional results are given in table 1.

It has been noted previously that in the case of a dye which is basic, toxicity is augmented with certain exceptions, by increase of OH in its

<sup>47</sup> Arch. Int. Med., 1913, 12, p. 613.

structure. It is claimed by some investigators that many dye-stuffs are more potent in a medium with reaction in the alkaline range than in one at or near neutrality and still more so when the hydrogen-ion concentration is low in the scale. Thus Prowazek<sup>48</sup> states that the addition of sodium carbonate increases the activity of methylene blue

TABLE 1  
DILUTION OF DYE STUFFS NECESSARY TO KILL *B. TYPHOSUS* IN 24 HOURS AT 37° C. IN  
VARIOUS MEDIA

Stain	Bile	Serum	Broth	Saline
Acridflavine .....	1,000,000	100,000	10,000	10,000
Auramine .....	1,000	1,000	1,000	10,000
Basic fuchsin .....	<1,000*	1,000	1,000	10,000
Brilliant green .....	10,000	<1,000	<1,000	<1,000
Crystal violet .....	<1,000	1,000	<1,000	1,000
Malachite green .....	<1,000	1,000	1,000	1,000
Methylene blue .....	<1,000	1,000	<1,000	1,000
New fast green, 3B.....	10,000	1,000	10,000	100,000
Proflavine .....	10,000	100,000	10,000	100,000
Pyronine G .....	<1,000	1,000	1,000	10,000
Rose bengal .....	<1,000	<1,000	<1,000	1,000
Safranin .....	<1,000	<1,000	<1,000	1,000
Spiller's purple .....	<1,000	1,000	<1,000	10,000

\* < signifies "less than."

TABLE 2  
EFFECTS OF CHANGES IN PH ON BACTERICIDAL ACTION OF CERTAIN STAINS ON *B. TYPHOSUS*  
IN 24 HOURS AT 37° C. FIGURES ARE DILUTIONS

Stain	Medium	PH	Results
Crystal violet.....	Bile .....	8.0.....	<1,000
Crystal violet.....	Bile .....	8.4.....	10,000
Crystal violet.....	Bile .....	8.8.....	100,000
Crystal violet.....	Bile .....	9.2.....	1,000,000
Crystal violet.....	Saline .....	4.8.....	1,000
Crystal violet.....	Saline .....	7.2.....	100,000
Crystal violet.....	Serum (inactivated).....	8.0.....	1,000
Crystal violet.....	Serum (inactivated).....	8.6.....	10,000
Acridflavine .....	Serum (inactivated).....	8.0.....	100,000
Acridflavine .....	Serum (inactivated).....	8.6.....	1,000,000
Brilliant green.....	Bile .....	7.9.....	<1,000
Brilliant green.....	Bile .....	8.7.....	<1,000
Brilliant green.....	Bile .....	9.2.....	1,000,000
Basic fuchsin.....	Bile .....	8.3.....	<1,000
Basic fuchsin.....	Bile .....	8.6.....	10,000
Basic fuchsin.....	Bile .....	9.1.....	100,000
Acid fuchsin.....	Bouillon .....	7.3.....	<1,000
Acid fuchsin.....	Bouillon .....	6.6.....	<1,000
Acid fuchsin.....	Bouillon .....	5.6.....	<1,000
Acid fuchsin.....	Bouillon .....	4.9.....	10,000

and Traube<sup>38</sup> finds that the same salt accentuates the toxicity of certain other stains, notably crystal violet. Graham-Smith<sup>37</sup> learned that the efficiency of homoflavine becomes greater by increase in alkalinity. Browning, Gulbransen and Kennaway<sup>49</sup> obtain some remarkable results in this direction with diamino-acridine methyl chloride, since they show

<sup>48</sup> Arch. f. Protistenk., 1910, 18, p. 221.

<sup>49</sup> Jour. Path. & Bacteriol., 1919, 23, p. 106.

that the sterilizing effect of this compound is multiplied one hundred times by a change from  $P_H$  4.0 to  $P_H$  11.0.

A few experiments in this direction were attempted, but since application of results to the problem at hand seemed overdiffficult, they are fragmentary. Their outcome is presented in table 2. Each test was carefully controlled by a check tube with reaction altered according to the series which it accompanied, but with no dye added to it. In all cases the controls were positive at the expiration of the period.

It is of interest to note that confirmation of the work of Browning and those associated with him regarding the exalted potency of acridine compounds in serum is found. Some dye-stuffs undergo an increase in toxicity toward typhoid with a rise in the  $P_H$  value and agreement in this detail is made to the statements of Traube,<sup>38</sup> Graham-Smith,<sup>37</sup> of Browning, Gulbransen and Kennaway<sup>49</sup> and others. One acid stain observed, acid fuchsin, shows the reverse of the above since it becomes increasingly toxic as hydrogen-ion concentration advances.

#### PREPARATION OF EXPERIMENTAL CARRIERS AND PROOF OF THE CARRIER STATE

Having ascertained by the survey just outlined which coloring matters are most toxic to *B. typhosus* in serum and in bile, the next step was to examine their potency in animals prepared as experimental carriers of this organism. The work of Gay and Claypole<sup>47</sup> and followed later by Stone<sup>50</sup> has resulted in providing a reliable technic for the production of such experimental animals. The outline of this method is as follows:

The organism which is known as *B. typhosus* 3 in the records of this department was cultivated on 5 or 6% rabbit-blood agar slants. The tubes picked out with care were 18 mm. in diameter, and the surface of the slant was as long as possible without moistening the plugs. Heavy and widespread inoculation was made and the tube incubated for 20-24 hours at 37 C. The dose which was administered intravenously in the posterior ear vein was  $\frac{1}{3}$  of such a culture suspended in physiologic salt and made up to such dilution that the volume in each instance was 2 c.c. Rabbits with weights between 1,500 and 2,000 gm. were selected. Animals thus treated lose a large fraction of weight which may amount even to 20% during the first 48 hours, and some of them die from the effects. Ordinarily, however, if the culture

<sup>50</sup> Jour. Infect. Dis., 1919, 25, p. 284.

has not been transferred too often previously on blood agar, such loss will not be prohibitive. Subsequent treatment was not given until the rabbits had commenced again to gain in weight, as shown by daily readings on the scale. This interval is from 8 to 14 days.

By the method here outlined, Gay and Claypole record 74% carriers and Stone approximately 94%. Although Nichols<sup>19</sup> succeeded at first in obtaining only 28%, he shortly afterward published results<sup>20</sup> which indicated that he must finally have succeeded in the majority of cases. Weinfurter<sup>51</sup> has shown that the percentage of positive animals varies with the strain utilized, which may account for failures.

In this work it was felt to be necessary that there be controls since proof cannot be presented that certain animals which had received treatment and which at the time of necropsy were negative for typhoid in the gallbladder, previously had been carriers. Therefore necropsy examinations of all animals which died during preparation or which have been killed for various purposes have been made promptly, and cultures have been made from the bile. No animal was considered which had been injected less than one week before the necropsy examination. This list includes 13 animals, all of which were proved to be typhoid carriers. Additional proof of the worth of the method under conditions obtaining at the time of the series thus is given. Such proof is of most vital importance for consideration of the latter portion of this work.

Fecal examination as a method of determining that a rabbit is a carrier following this method of injection is worthless.

But a small number of positive tests, 3 out of 23, were obtained by the use of plates of Harris and Teague's<sup>52</sup> eosin-methylene blue medium. Negative tests followed trials with the brilliant green-eosin agar of Teague and Clurman,<sup>53</sup> the liquid brilliant green formula of Browning, Gilmour and Mackie,<sup>54</sup> the technic of Browning, Mackie and Smith,<sup>55</sup> wherein potassium tellurate is added to brilliant green broth, and of Endo plates. Yet subsequent necropsy examination proved the animals to be positive carriers.

In spite of the fact that it is generally recognized that rabbits are most refractory to the use of cathartics, elaterin was tried. Tonney, Caldwell and Griffin<sup>56</sup> have stated that this compound simplifies and renders more certain the procedure of detection of *B. typhosus* in human feces. Bearing their work in mind, therefore,  $\frac{1}{42}$ th grain of elaterin was administered to a rabbit. Aside from causing the animal to be more decidedly dull for a few hours, there were

<sup>51</sup> *Centralbl. f. Bakteriologie*, I, O., 1914, 75, p. 379.

<sup>52</sup> *Jour. Infect. Dis.*, 1916, 18, p. 596.

<sup>53</sup> *Ibid.*, p. 647.

<sup>54</sup> *Jour. Hyg.*, 1913-14, 13, p. 335.

<sup>55</sup> *Jour. Path. & Bacteriol.*, 1914-15, 19, p. 127.

<sup>56</sup> *Jour. Infect. Dis.*, 1916, 18, p. 239.



no effects. It cannot be used for such animal, therefore. Thus, fecal examination for the detection of *B. typhosus* in the feces of rabbit carriers by methods ordinarily in use yields most unsatisfactory results. Cathartics likewise are valueless.

Some of the animals used in this series of experiments were subjected to laparotomy and a sample of the bile aspirated with a sterile syringe. The presence of *B. typhosus* was then determined.

The persistence of the experimental carrier state in rabbits is well recognized. Doerr<sup>57</sup> found animals to be infected 120 days after inoculation and Johnston,<sup>58</sup> 110 days.

Therefore the argument cannot be advanced with certainty that the condition of animals about to be considered had cleared up spontaneously during the period between operation and subsequent treatment with dyes.

#### TREATMENT OF EXPERIMENTAL CARRIERS WITH DYE STUFFS

Five coloring matters, auramine (pyoktannin aureum), acriflavine, proflavine, pyronine G and new fast green 3B showed bactericidal action in bile and in serum. According to plan, these were administered intravenously to rabbits. Preliminary tests were made to determine their toleration by the animals.

(a) Auramine was first utilized. This material, which was first described by Stilling,<sup>59</sup> has been advocated for external use. A saturated solution in physiologic salt, which is less than 0.5% when administered intravenously in the amount of 1 c c, was followed by great increase in the rate of respiration to 175 per minute. This persisted for one half hour. The compound, therefore, is too toxic for purposes of intravenous injection.

(b) Flavine compounds have attracted an immense amount of attention during the past six years because of their adaptation to war purposes for irrigation and dressing of wounds. Originally elaborated by Benda for experimental use with protozoan infections, they have been adopted widely for germicidal use. Their high efficiency has been established by Browning<sup>60</sup> and his co-workers and by Morgan.<sup>44</sup>

In large measure they are unique in their compatibility toward serum, as found by Browning and those associated with him who claim that serum increases their toxicity toward various organisms over tenfold in comparison to their activity in peptone water. Mueller,<sup>45</sup> with live cultures of tissues, did not confirm this. Gay and Morrison<sup>26</sup> have demonstrated an increase of germicidal

<sup>57</sup> *Centralbl. f. Bakteriologie*, I, O., 1905, 39, p. 624.

<sup>58</sup> *Jour. Med. Res.*, 1912, 27, p. 177.

<sup>59</sup> *Anilin Farbstoffe als Antiseptica und ihre Anwendung in die Praxis*, 1890.

<sup>60</sup> *Jour. Path. & Bacteriology*, 1919, 22, p. 265; *Jour. Hyg.*, 1919, 18, p. 33; *Jour. Path. & Bacteriology*, 1919, 23, p. 106.

activity of acriflavine in the presence of pus. Browning and his co-authors have been responsible for urging the utility of this series of compounds for practice in wound treatment, although objections somewhat strenuous have been entered by Fleming<sup>61</sup> and more conservative negative criticism has been voiced by Hewlett<sup>62</sup> and by Pearson.<sup>63</sup> These combinations are shown to be tolerated comparatively readily by animals by Browning and Gulbransen<sup>64</sup> and by Taylor and Austin.<sup>65</sup>

Of the most pertinent interest also are the experiments which have been carried out regarding chemotherapeutic possibilities of acridine compounds. Although Browning<sup>66</sup> did not recommend flavine for action at a distance from its point of application, with Gulbransen<sup>67</sup> he found later that when injected into the animal it causes serum to become germicidal toward *B. coli* and *Staphylococcus aureus*, and these men suggest its possible application as a bile bactericide. Byam, Dimond, Sorapure and Wilson<sup>68</sup> made use of acriflavine intravenously in cases of trench fever with results which they thought to be beneficial, and Hussey<sup>69</sup> states that acridine compounds with gold, silver or cadmium have marked inhibitory powers for streptococci, *B. anthracis*, gonorrhea and polyarthritis. Neufelt and Schiemann<sup>70</sup> used acriflavine and proflavine on mice, some injected with pneumococcus and others with chicken cholera and as a result believed that the onset of these diseases was delayed a number of hours. A portion of the dye possibly is excreted through the bile, although Davis and White<sup>71</sup> report that the urine after injection becomes germicidal toward certain micro-organisms.

With these reported facts in mind, therefore, and with the results indicated in table 1 as an immediate basis, acriflavine and proflavine were experimented with as follows:

*Acriflavine*.—Rabbit 671 had been shown previously to be a carrier of the typhoid bacillus through fecal examination. By intravenous injection, were administered 10 doses each of 2 cc of 1% acriflavine in sterile physiologic salt solution. Little reaction was evident other than slight irritation shortly following administration and a small amount of sloughing at the site of injection some days later. Doses were given generally 2 days apart. This animal was then killed by exsanguination and a necropsy examination made. Examination showed the intercostal muscles to be heavily stained with the dye. There was evidence of slight coccidiosis in the liver. The gallbladder contained 1.5 cc of light green bile, which was clear except for a few fine floccules suspended within it. Although streaks were made on agar plates from the bile, blood, urine, liver, spleen and kidney, after proper incubation growth developed only from the bile. This was copious and agglutinated readily with antityphoid serum at 1:100.

<sup>61</sup> Lancet, 1917, 193, p. 508.

<sup>62</sup> Lancet, 1917, 193, 727.

<sup>63</sup> Lancet, 1918, 194, p. 370.

<sup>64</sup> Proc. Roy. Soc., 1918, 90, p. 136; Jour. Hyg., 1919, 18, p. 33.

<sup>65</sup> Jour. Exper. Med., 1918, 27, p. 635.

<sup>66</sup> Lancet, 1917, 193, p. 436.

<sup>67</sup> Proc. Roy. Soc., 1918, 90, p. 136; Jour. Path. & Bacteriol., 1919, 22, p. 265.

<sup>68</sup> Jour. Roy. Army Med. Corps, 1917, 29, p. 560.

<sup>69</sup> Ztschr. f. Geburtsh., 1918, 80, No. 2.

<sup>70</sup> Deutsch. med. Wchnschr., 1919, 45, p. 844.

<sup>71</sup> Jour. Urol., 1918, 2, p. 299.

Thus it appears that acriflavine given intravenously is useless for clearing up rabbit carriers of typhoid.

It was then determined to learn whether acriflavine is of any use for the purpose stated if administered subcutaneously. Rabbit 677, therefore, was given four 2 c.c. injections of 1% acriflavine in physiologic salt solution over the abdomen. This animal likewise had been proved by fecal examination to be a typhoid carrier. Following this series, the animal, which was in very poor condition, was exsanguinated and a necropsy examination was made. Examination revealed dry necrosis in the areas of injection. Of the musculature, the intercostals especially were intensely colored by the dye. The gallbladder was greatly distended with 3 c.c. of a very light green bile containing much sediment. Again streaks were made on agar plates from the bile, blood, liver, spleen, kidney and urine. Twenty-four hours' incubation developed a heavy growth from the bile which by agglutination was proved to be *B. typhosus*.

Thus it was indicated that acriflavine is worthless when given either intravenously or subcutaneously for the purpose of sterilizing infected gallbladders of experimental animal carriers of typhoid.

*Proflavine*.—The animal, 675, had been proved to be a typhoid carrier. To it were given intravenously in the ear three 5 c.c. doses of proflavine made up to 1% in physiologic salt solution. No reaction from this treatment either immediately or afterward was apparent. These injections were spaced to 2 or 3 day intervals, and the rabbit was killed by exsanguination 2 days after the end of the series. Necropsy examination showed that the gallbladder contained 1 c.c. of bile with normal appearance. Streaks made on agar plates from the bile, blood, liver, spleen and kidney were all negative for *B. typhosus* after 24 hours' incubation. The muscles were much less colored following the administration of proflavine than they had been in the other animals after the use of acriflavine. As a period of nearly 2 months had elapsed between the time of preparation of the animal and proof that it was a carrier and the period of treatment, it was deemed wise to repeat this work with additional animals.

Rabbit 657 was then treated with a series of injections like that previously administered to 675 except that the aggregate amount was 14 c.c. instead of 15 c.c., the first dose being only 4 c.c. Two days after completion of the treatment, 657 was exsanguinated and examined. There was no gallbladder. Streaks were made from the blood, liver, spleen and kidney and after incubation, an abundant growth of *B. typhosus* was obtained from the liver only. As this animal remained a carrier after proflavine, the series of tests was carried no further. It was felt that proflavine is ineffective for the purpose sought.

(c) Pyronine G, while comparatively inactive in bile, was toxic to *B. typhosus* in serum at 1:1,000. It was deemed to be of sufficient importance to warrant a test in vivo.

Rabbit 620 was given intravenously 5 c.c. of 1% pyronine G in physiologic salt solution in the ear vein with no unfavorable reaction following it. Two days later it received 2 c.c. of the same solution, and 48 hours afterward was exsanguinated. The gallbladder contained 1.6 c.c. of light green, clear bile. Cultures made from this bile and from the usual tissues were negative for *B. typhosus*.

Two more rabbits, 616 and 662, were each injected with 4 c c of 1% pyronine G in physiologic salt solution, while 636, which was in much poorer condition, received 3 c c. Contrary to experience with 620 mentioned in the preceding paragraph and also with another animal which had been used in preliminary tests to determine the relative tolerance of rabbits to pyronine G, each of these showed much excitement after the dose and in about one minute uttered shrill cries. These symptoms subsided in a few moments, but the day following all of the rabbits were very dull. Forty-eight hours after the injection 616 was paralyzed in the hind quarters, and 4 days later it was killed. The gallbladder was greatly enlarged, containing 3 c c of grayish and very turbid bile. From streaks made on agar plates from the bile, blood, liver, spleen and kidney, heavy growth of *B. typhosus* developed from the bile.

The other animals were not killed at once, as it appeared that the results given in the foregoing were conclusive. Although one animal was shown to be negative for *B. typhosus* after treatment, it was barely possible that it was not infected by the original injection of *B. typhosus*. Subsequent necropsy examination of 662 following another experiment showed that it too was highly infected with the typhoid organism. When the bile from 616 was shaken out with chloroform, no pink tint of pyronine G appeared. It is therefore suggested that this dye is not excreted through the bile. A similar test with the urine gave a positive color to the chloroform. Pyronine G thus was shown to be useless as an agent for clearing up the condition of typhoid carrier rabbits.

(d) The germicidal activity of new fast green 3B toward *B. typhosus* is 1:10,000 in bile and 1:1,000 in serum. This dye stuff belongs to the triphenylmethane group according to Green's classification. It is described as very soluble in hot water, but only sparingly so in cold. The presence of an electrolyte alters the condition of a solution of it markedly, since when the salt is present even in small amounts, the tendency is for a gel to be formed. On the other hand, in distilled water, the state is that of a sol. Evans and Schulemann<sup>72</sup> indicate that a dye stuff which is colloidal is less diffusible when introduced into the body tissues, and that it is more toxic to the animal. Evidence of toxicity did appear in some of the animals. Judging by a series of observations on this subject, the dye when introduced into the circulation tends to form emboli which may lodge in the brain. Ordinarily, however, when made up into a 0.5% solution in distilled water and injected somewhat slowly, a rabbit weighing 2,000 gm. can tolerate 3 c c easily. It is interesting to note at this point that a rabbit injected with this coloring matter is likely to show for 4 or 5 minutes afterward the anaphylactoid symptoms which have been described in recent articles

<sup>72</sup> Science, 1914, 39, p. 443.

by Hanzlik and Karsner,<sup>73</sup> following introduction of certain colloids and arsenicals into the circulation. Repeated intravenous injection of new fast green 3B results also in increased fragility of the arteries, as became evident when the ordinary technic of bleeding from the carotid was practiced. The walls of the blood vessel tear very easily. In addition, its use intraperitoneally is followed by edema over the abdomen, which is especially evident in the axillae. Likewise, adhesions result.

New fast green 3B is secreted through the bile in large amounts in the guinea-pig and to a less degree in the rabbit. It is a simple matter to make up a series of color comparates by adding to bile in known quantities in tubes the dye-stuff in certain dilutions. If, then, these mixtures in bile are shaken out with a certain portion of chloroform, a small part of thymol added and the whole overlaid with a layer of paraffin oil, we obtain a series of color comparates which may be used for purposes of comparison with unknowns for some days if kept out of direct light when not needed. In one instance in the guinea-pig the concentration of the coloring matter within the bile 5 minutes after intrajugular injection was shown to be 1:6,000 by this method. The animal died suddenly 3 minutes after treatment.

*The Elimination of New Fast Green 3B Through the Bile in the Guinea-Pig Following Intraperitoneal Injection.*—A guinea-pig, weighing 450 gm. received 3 c c of 0.5% solution of new fast green 3B in distilled water. The animal became sick in 4 or 5 minutes and fell over on its side but recovered. Five hours later, it received 4 c c more of the dye solution. It became very weak. It was chloroformed and a necropsy examination was made one and three quarters hours afterward. The wall of the peritoneal cavity was stained heavily, as were all of the abdominal organs, but there was no stain elsewhere in the tissues. The bile when shaken out with chloroform indicated that the concentration of the stain was approximately 1:10,000.

Elimination of new fast green 3B through the bile in the rabbit appears to take place in varying degree. One rabbit which had received an intravenous injection of 4 c c of a 0.5% solution was exsanguinated and necropsy examination performed 2 hours later. The bile showed the dye-stuff in a concentration of approximately 1:6,000. Two other rabbits proved to have a 1:10,000 dilution of the stain in the bile following a similar technic. When the stain is introduced intraperitoneally into this animal, it is found in the bile in traces only as was proved by two attempts. Possibly a difference in the structure of the

<sup>73</sup> Jour. Phar. & Exper. Therap., 1920, 14, p. 379.

lymphatic system in the guinea-pig and the rabbit may account for this variation in the rate of secretion of the stain.

Bile to which new fast green 3B has been introduced in vivo is germicidal to *B. typhosus*. Three animals which died shortly after injection of this stain gave high concentrations of the dye in bile at the time of necropsy. Two of these were guinea-pigs, one of which gave an indicated dilution of 1:10,000. When a 2 mm. loop of a 24-hour broth culture of *B. typhosus* was added to these samples of bile and the mixtures then incubated, it was shown by streaking out on agar plates that the organisms were killed within 5 hours. In check rabbit bile, the micro-organism will live for months.

The following protocols indicate the results produced by injection of new fast green 3B in an endeavor to clear up experimental rabbit carriers of typhoid.

*New Fast Green 3B in Experimental Rabbit Carriers.*—No. 346 was given intravenously an aggregate dosage of 4.5 cc of 1% new fast green 3B in physiologic salt solution within a 7-day period. No unfavorable reactions resulted. Two days after the final injection, the animal was exsanguinated. Necropsy examination revealed the muscular tissues to be tinted slightly by the dye-stuff. The bile, 0.75 cc in amount, was light green and clear. Streaks were made on agar plates from the bile, blood, liver, spleen and kidney. After proper incubation, there was no growth.

No. 660 received intravenously 5 cc of 1% new fast green 3B in total amount during 7 days. No unfavorable symptoms appeared. Five days after this series was complete, the rabbit was killed by bleeding to death. Examination showed the gallbladder to be very small and to contain  $\frac{3}{16}$  cc of bile which was clear, light green tinted with yellow. The walls of the gallbladder were somewhat thickened and roughened. Smears made on agar from the bile, blood, liver, spleen and kidneys were all negative for *B. typhosus*. The bile showed a  $P_H$  of 8.4, which is much higher than that found in the normal rabbit by the author<sup>74</sup> in a previous communication, and which of itself may indicate that the animal recently had been a carrier.

No. 662 was injected intravenously with an aggregate amount of 5 cc of this dye-stuff in 1% concentration in physiologic salt solution over a period of 7 days. Again no symptom was unfavorable. It was exsanguinated 3 days after the final dose had been administered. Necropsy examination revealed a very small gallbladder containing 0.2 cc of light green and very clear bile. Its walls were decidedly thickened and roughened inside. Smears were made on agar plates from the bile, scrapings from the gallbladder wall, spleen, kidney, liver and blood. Growth rather sparse from the bile and heavy from the scrapings appeared and was later identified as *B. typhosus*.

No. 307 received intravenously 7 cc of 1% new fast green 3B in physiologic salt solution during a period of 6 days with 2 days between injections. Twenty-four hours following the termination of this series it was bled to death from the carotid. Necropsy examination revealed no trace of the stain except in the bile which when shaken out with chloroform yielded a slight tint of the

<sup>74</sup> Proc. Soc. Exp. Biol., 1920, 28, p. 36.

dye-stuff. The gallbladder contained 0.75 cc of light greenish yellow and somewhat turbid bile. Streaks were made from this sample of bile and after 24 hours' incubation, there appeared a small amount of *B. typhosus*. The condition of this animal, therefore, at least was not cleared up entirely by treatment with new fast green 3B, although the intense reaction and the extreme emaciation which followed the original reaction would indicate a most thoroughly infected host. The weight of the animal increased markedly during treatment.

No. 313 received in the ear vein 1.5 cc of 1% new fast green 3B in physiologic salt solution. Although no unfavorable reactions were manifest, this animal was found dead the following morning. Necropsy examination showed that the fascia and connective tissues were deeply impregnated with the stain. The walls of the blood vessels likewise were highly colored with it. The gallbladder walls were definitely tinted, and the bile likewise gave a positive reaction after shaking out with chloroform. The bile, which amounted to 0.2 cc, was turbid and reddish with all the gross appearance of that of a typical rabbit typhoid carrier. Streaks were made on agar plates from the bile, blood, liver and kidney, but after incubation all were negative for *B. typhosus*.

No. 311 was injected intravenously with an aggregate amount of 11 cc of new fast green 3B in physiologic salt solution. The period was 8 days and 2 days elapsed between injections. The final dose was 4 cc, and this was followed by no discernible reaction. When exsanguinated 24 hours after the finish of the series, no trace of the stain was to be seen, and this time only a questionable trace of the dye-stuff could be determined by shaking out with chloroform. The 2 cc of bile was light green and clear. Streaks made from it on agar gave no sign of *B. typhosus*.

Rabbit 337 was prepared as an experimental carrier of typhoid, and 13 days later was operated on for laparotomy, the purpose being to remove by aspiration a portion of the bile in order to prove absolutely the carrier condition of the animal. This operation was performed without incident, and *B. typhosus* was found in quantity. After recovery, this rabbit received a total of 14 cc of a 0.5% solution of new fast green 3B in distilled water, extending over a period of 10 days. Aside from some signs of weakness following each injection, there were no untoward symptoms. On the third day after this series, the animal was exsanguinated. Necropsy examination showed that the wound at the site of operation had healed well. There were no adhesions. The gallbladder was of normal size and was filled with greenish bile. On aspiration with a syringe, it was found that the bile was decidedly mucous and therefore thick in consistency. Smear preparations made from it showed no organisms nor leukocytes present. A portion of the bile also was streaked out on agar plates, and tubes of beef bouillon were inoculated. All preparations were negative for *B. typhosus* after proper incubation. This animal heretofore had been sterilized of the typhoid between the time of operation and necropsy.

No. 340, a large animal, weighing 3,500 gm., was given  $\frac{1}{2}$  slant on *B. typhosus* grown on blood agar. This was followed by a strong reaction and slow recovery. Twenty-four days later, while still greatly emaciated, it was injected intravenously with 1 cc of a 0.5% solution of new fast green 3B in distilled water. This solution had been centrifugalized just previously to remove possible particles from it. At 3 minutes and at 50 minutes after introduction of the stain, the rabbit fell over momentarily and showed spasmodic effects, but the following day seemed to have recovered. The following day it

received 0.75 c.c. of the same solution of the dye, and in 25 minutes expired quietly. At the necropsy examination, which was made immediately, the blood vessels in the liver were noted to be distinctly colored. The bile, which was in a gallbladder greatly enlarged, was light green, very turbid and somewhat mucous. The cloudiness was of unorganized material. A portion of this bile was streaked out on agar plates and some added to bouillon. All preparations were positive for *B. typhosus* after incubation and agglutination. This animal, therefore, which was very large and which had received but an exceedingly small portion of the stain before its death, was not sterilized in the gallbladder.

No. 319 was operated on 11 days after injection of *B. typhosus* and by aspiration of bile from the gallbladder was proved to be an experimental carrier through growth of the micro-organism and its subsequent agglutination with antityphoid serum. It received 9 c.c. of a 0.5% solution of new fast green 3B in physiologic salt solution in two doses and at 2-day intervals. Death took place suddenly 29 minutes after injection of a third portion of 5 c.c. of the same solution. This solution had been centrifugalized just before using in order to remove small particles possibly present in suspension. Necropsy examination revealed adhesions between the omentum and peritoneum. A slight tint of color in the cortex of the kidney was the only trace to be found in the body. The gallbladder contained 0.5 c.c. of grayish bile which, after planting in bouillon, yielded *B. typhosus*. With the typhoid likewise were many staphylococci. Evidently the coccus had produced a secondary infection.

Rabbit 427 by operation 13 days after typhoid injection was proved to be a gallbladder carrier of the micro-organism. During a period of 10 days it received an aggregate of 12 c.c. of 0.5% new fast green 3B in distilled water. Twenty-four hours later it was bled to death. It was found at necropsy that the lower portion of the liver was adherent to the abdominal wall, with the gallbladder embedded in tissue. The bile was light green and with a little mucus in it. From streaks made from this bile on agar plates, growth of *B. typhosus* appeared.

By experiments already described, it seemed evident that when administered intraperitoneally in the rabbit, new fast green 3B is not excreted readily through the bile. As it appeared to be a possibility that even though not present in the bile in a large amount it might have a sterilizing effect, it was decided to make an attempt to clear up a known carrier by intraperitoneal injection of the dye-stuff. The protocol follows:

*The Effect of New Fast Green 3B by Intraperitoneal Injection on a Known Carrier.*—No. 628 was a known carrier having already been operated on to prove the fact. The animal received 7.5 c.c. of a 0.5% solution of the dye-stuff in distilled water, and 24 hours later the series, which had included 3 days, was bled to death. The gallbladder was enlarged and contained a light yellowish bile. This sewn into tubes of bouillon, developed *B. typhosus*. No trace of the stain was evident in the areas of injection, having diffused little. Edema was extensive over the abdomen and was especially evident in the axillae. This method of treatment, therefore, is ineffective for the purpose sought.

As immediate checks for the series of treated rabbits just described, 2 animals, 314 and 316, were used. They were utilized in connection



with Nos. 307, 311 and 313, at the same time and with a like preliminary dosage of *B. typhosus*. They were killed at the same time as 307 and 311. At necropsy each was found to be a positive carrier of the micro-organism. It should be noted that these animals were additional to the 13 previously mentioned.

It is therefore evident that new fast green 3B sometimes kills the animal, apparently from embolus formation. If some method were elaborated whereby this tendency to flocculate in the blood stream might be obviated, this difficulty should be eliminated. Attempts to bring about this result with protective colloids were failures. New fast green 3B placed in normal inactivated rabbit serum so as to make a concentration of the stain  $\frac{1}{4}\%$  and then left at room temperature for 24 hours precipitates partially. If this solution after such time be centrifugalized at high speed and the supernatant then injected into a normal guinea-pig of 700 gm., it appears that the toxicity of the mixture has been exalted. The animal dies suddenly. In this instance only 2 c.c. of this solution were injected by the intrajugular method. Death took place in 3 minutes. Attempts to use 6% gelatine in distilled water and 7% gum acacia also in distilled water with new fast green 3B in concentration of 0.5% in these gave comparable results with rabbits. Death occurred within from 3 to 5 minutes. The symptoms were anaphylactoid.

In this series of animals, 10 rabbits were treated intravenously with new fast green 3B. When examined later, 5 were negative for *B. typhosus* in the bile. Of the 5 which were positive after treatment intravenously, one showed only a small number of micro-organisms, indicating that possibly it might have been in process of clearing up. Another one of those positive died before receiving more than a trace of the dye-stuff. Some of the animals had undergone laparotomy in order to prove them carriers. Carrier rabbits rarely clear up spontaneously. Two animals which had received no stain injections were used as immediate checks against the 6 of the series which had not been operated on in order to prove them positive carriers of *B. typhosus*, and these were in addition to the 100% (13) animals previously noted.

#### SUMMARY

*The Bactericidal Action of Dye-Stuffs.*—The two acridine compounds, acriflavine and proflavine, are more germicidal in the presence of serum than when it is lacking. This is nearly unique.

In most instances, bile depresses the activity of stains fully as much as serum.

The germicidal effect of many stains is increased by lowering hydrogen-ion concentration, while in the case of at least one stain, acid fuchsin, the reverse is true.

*Experimental Rabbit Carriers of Typhoid.*—The percentage of experimental carriers of typhoid which may be produced successfully by the Gay-Claypole technic is very high with the condition of the culture as it was at the time of its use in this series. Of those animals which survived one week, 100% were carriers.

Attempts to detect *B. typhosus* from the feces of experimental rabbit carriers of typhoid are wholly unsatisfactory. Either the organisms are not present, are attenuated or are dead by the time they have reached the rectum. Elaterin is useless as a cathartic to increase the percentage of positive findings.

*Chemotherapeutic Results.*—Auramine, acriflavine, proflavine and pyronine G, although active *in vitro* against *B. typhosus*, are ineffective as agents for sterilizing gallbladders, containing *B. typhosus* in the living body. The first is too toxic to the host. The others are excreted through the urine rather than through the bile.

In certain instances new fast green 3B offers possibilities as a germicide *in vivo* for *B. typhosus* in gallbladders of experimental rabbit carriers. It retains its activity in serum and in bile. It is excreted through the bile when administered intravenously. Bile taken from animals treated with this dye and killed shortly after is germicidal to *B. typhosus*. This dye-stuff is not effective in clearing up the condition of all animals. It may be very toxic to the animal. It changes readily from the sol to the gel state, apparently dependent on the presence of an electrolyte. The result is the formation of emboli. Attempts to remove these objectionable features by use of other colloids have been failures. It is without germicidal effect on infected gallbladders of the rabbit if injected intraperitoneally.

# ORGANISMS OF B. LACTIMORBI GROUP FOUND IN THROAT CULTURES AND SIMULATING B. DIPHTHERIAE

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Some years ago, before the routine of tests for virulence had been established, complaints were received that several patients were being quarantined for diphtheria for unusually long periods. Examination of the eighteen-hour serum cultures from the nose and throat showed a rod form with bipolar metachromatic granules, and with marked tendency to parallel arrangement. The organism was isolated, and it was at once clear that we were dealing with a form which did not belong even to the diphtheria group, as it was motile in the hanging drop and showed terminal spores in older cultures. The patients were discharged from quarantine and a brief note was presented to the Association of Pathologists and Bacteriologists in 1907 or 1908. It was our intention to carry out detailed investigation, but the cultures were lost and for a time no more such organisms appeared. In 1910 they were again noticed, but the morphologic distinctions were understood by the diagnostician and the patients were not isolated. For various reasons no further work was done other than to note the constant recurrence of the organism in cultures from various parts of the town, but the frequency of this recurrence, and the fact that the organism so closely resembles diphtheria and has not hitherto been described in the throat, makes a brief report valuable.

In the meantime the work of Jordan and Harris<sup>1</sup> had appeared, and their description of an organism found in association with milk sickness, and named by them *B. lactimorbi*, agreed closely with my own earlier findings. Later work by these investigators showed that the organism was probably from soil and had no relation to the disease, but the name is now established.

A detailed description on the official blanks of the Association of American Bacteriologists has been made, but the work will not be taken up in detail further than concerns the essential points, together with the group number.

Received for publication June 16, 1921.

<sup>1</sup> Jour. Infect. Dis., 1909, 6, p. 401.

## GROUP NUMBER—211.3333533

There are evidently several strains, which differ mainly in degree, some having a tendency to long forms and some to short forms, some showing earlier spore formation than others, and so forth, but so far none have been found which did not conform to the group number as noted above.

*Morphology.*—There are rods with about the same size and the same variations as the C type of diphtheria in Wesbrook's classification and

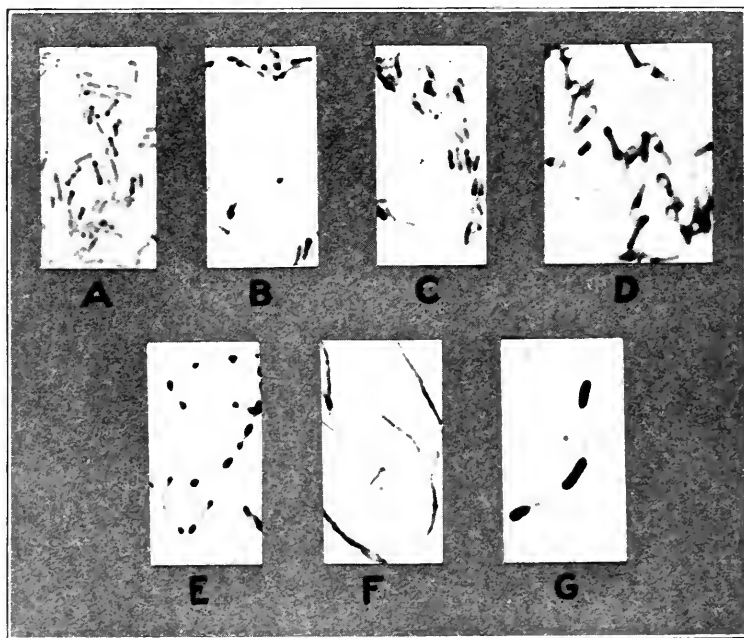


Fig. 1.—A, pure culture, 1911,  $\times 1500$ , 18 hours, blood serum; B, pure culture, 1921,  $\times 1500$ , 18 hours, blood serum; C, throat culture, 1921,  $\times 1500$ , 18 hours, blood serum; D, spores, 1921,  $\times 2000$ , 30 hours, glycerine agar; E, involution (?), 1921,  $\times 1500$ , 48 hours, glycerine agar; F, involution, 1921,  $\times 1500$ , 20 days, gelatin; G, involution, 1921,  $\times 1500$ , 30 days, milk. With the exception of A, all were stained with methylene blue (Loeffler's), diluted 1:50 with water, for from 4 to 10 seconds. Photographed by the method described by Charles F. Brush in *The Physical Review*, 1910, 31; average exposure, 20 seconds on panchromatic plate, development 9 minutes.

containing deeply stained metachromatic granules. These granules are usually bipolar but may be unipolar, or even central. The granules are usually of the same diameter as the cell, and do not cause the apparent bulging usually noted in diphtheria. This is so characteristic that as soon as one becomes accustomed to the organism, it can readily be

distinguished in the routine throat cultures. When it is present in association with true diphtheria, differentiation is more difficult. The intergranular space stains lightly, and with special stains such as Albert's, both body and granules react in a manner identical with diphtheria.

There is a marked tendency to parallel arrangement, though perhaps the average number per bundle is less than with diphtheria.

On favorable mediums such as fresh blood serum, there are frequently no spores in the first 24 hours, while on dried out tubes these naturally appear earlier. The spores are almost always terminal, much like those in tetanus, but somewhat more oval, and about double the diameter of the cell. There is usually a terminal granule at the base of the spore, but occasionally the spore develops slightly more centrally, pushing the granule ahead of it to the extreme tip. The spores stain readily by the usual methods.

The metachromatic granules develop early, and in all mediums except in broth, in which few granules are found, without any preliminary solid stage, so that four- or five-hour cultures have about the same morphology as those of from 15 to 20 hours. In old cultures, especially in gelatin and in milk, there are found mostly involution forms, with great irregularity of the staining material, and of the morphology, and strangely enough, scarcely any spores are seen. These cultures are viable, and the new generation conforms to type.

In the hanging drop there is active motility, and peritrichous flagella have been demonstrated. From the water of condensation of agar or from broth there is a marked tendency to long chains which move as a unit in undulating lines, the granules clearly visible on account of the difference in refractive index. Incidentally, it has been found that by the use of very dilute stains, a small amount of impregnation of the granules can be obtained before cessation of motility, but these faintly stained organisms are no longer viable and soon die if let alone. Gram's stain is negative, a further distinction from the Klebs-Loeffler bacillus.

The organisms are not acid-fast.

*Cultural Characteristics.*—The growth on the surface of solid mediums is not characteristic, resembling in a general way that of the colon group. On the chart the growth on agar is described as abundant, spreading, flat, glistening, smooth, translucent, butyrous, and yellowish in old cultures, but without notable odor. On blood serum it is about the same but usually rather more raised, probably because the medium is less fresh than the routine agar.

In broth there is a membranous pellicle, easily disturbed and sinking to the bottom, with a moderate cloud and an abundant sediment, viscid on shaking. In old cultures the broth usually becomes almost clear with a ball-like mass at the bottom, composed of the long involution forms noted in the foregoing. There is no odor and no pigment formation.

In gelatin the growth at room temperature is very slow, with a gradual funnel liquefaction, which tends to evaporate, leaving a cup-like depression. In gelatin at 37 C. there is complete loss of power to resolidify at the end of 48 hours, and the subsequent appearance of the culture is much like that of old broth.

Chemically the organism is inert. There is no fermentation of sugars, no diastatic action, no nitrate reduction. In milk there is a continuous alkaline reaction with peptonization, beginning about the seventh day at 37 C. and complete at about the tenth day. Blood serum is slowly liquefied, best seen when kept in a moist atmosphere to prevent drying. Luckhardt<sup>2</sup> found virulence in one strain, but was unable to find it in others, and so far none has been noted for small animals in this present series. No experiments in feeding have been made as there are no clinical symptoms connected with the presence of the organism.

*Frequency of Appearance.*—In the last 8 or 10 years they appear in rather less than 1% of the cases in the Cleveland Health Division Diagnosis Laboratory, and careful controls of mediums and other substances remove the possibility of their being a laboratory contamination. In 1920 they have also been found occasionally in normal throats in class culture work. When present in a throat they persist indefinitely and show no relation to clinical phenomena. They appear to be occasional saprophytic inhabitants of the upper air passages, and when their hosts become infected with diphtheria there is a period in which both species are present. The diphtheria bacillus then disappears, but the other persists, and unless the morphology is recognized, there is a distinct probability that the release cultures will continue to be reported as positive, until virulence tests are made.

We have also found the organism of great value in teaching, especially in the elementary work, in which the combination in one organism of motility, spores, metachromatic granules and harmlessness, is a great trouble saver.

<sup>2</sup> Jour. Infect. Dis., 1909, 6, p. 492.

The accompanying illustration shows the morphology alone and in mixed cultures, and requires no special explanation.

#### SUMMARY

In normal and pathologic throats organisms occur from time to time which under routine conditions so closely resemble *B. diphtheriae* as to cause possible confusion.

These organisms may be readily differentiated, even under these conditions, by certain constant morphologic characteristics, and when isolated spores and motility are seen.

# FURTHER OBSERVATIONS ON A RAPID METHOD OF PNEUMOCOCCUS TYPING

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This report is concerned with the experience obtained in a series of 100 consecutive, unselected cases of pneumonia in which typing of the pneumococcus from the sputum was made by (1) the rapid method described by the writer,<sup>1</sup> (2) the Avery method,<sup>2</sup> (3) a modified Avery method in which inulin is substituted for dextrose and an indicator is added to the medium, and (4) the mouse method, whenever possible.

## METHODS

*Rapid Method*<sup>1</sup>.—The rapid method I have employed is a rapid precipitin test on bile treated sputum, based on the solubility of the pneumococcus in bile. After a direct smear of the sputum has been made, from 1 to 2 c c of sputum are placed in a clean centrifuge tube. To the sputum is then added from 3 to 5 drops of undiluted ox bile (or a 10 % solution of sodium taurocholate) and a sufficient quantity of sterile physiologic sodium chloride solution, if necessary, to insure a specimen of sufficient fluidity to allow of centrifugation. The mixture is then thoroughly stirred and broken up with a glass rod. It is sometimes advantageous to effect the breaking up and mixing of the sputum, bile and salt solution by grinding in a small mortar with a pestle. The tube is then heated in a water bath at a temperature of from 42 to 45 C. for 20 minutes, which time suffices for a solution of the pneumococci by the bile. The fluid is then centrifugalized. Of the centrifugate, from 0.3 to 0.5 c c quantities are carefully pipetted into each of 3 small, scrupulously clean tubes. To the first tube is added from 1 to 2 drops of undiluted type 1 pneumococcus antiserum, and to the second and third tubes the same quantity of type 2 and type 3 antiserum, respectively. A positive precipitin test is evidenced by an almost immediate clouding and flocculation, which is enhanced by heating at 42 C. in a water bath for from 10 to 20 minutes.

Received for publication June 16, 1921.

<sup>1</sup> Jour. Infect. Dis., 1920, 27, p. 310.

<sup>2</sup> Jour. Amer. Med. Assn., 1918, 70, p. 17.



*Modified Avery Method.*—The rapid cultural method for the determination of types of pneumococcus, described by Avery,<sup>2</sup> consists in the use of a meat infusion broth, 0.3 to 0.5 acid to phenolphthalein, with 1 % glucose and 5 % rabbit blood. Recently, we have found that the substitution of 1 % inulin for the dextrose offers certain advantages. In addition to the 1 % inulin, Andrade indicator is added to the blood or ascitic fluid broth, P<sub>H</sub> 7.6.

Dextrose is broken down by both the streptococcus and pneumococcus. Inulin is attacked, with acid production, by the pneumococcus, whereas the streptococcus, with the exception of rare strains, fails to break down inulin. The presence of a fermentable carbohydrate in the medium appears to favor the growth of the pneumococcus, to the detriment of associated streptococci. If a pneumococcus is present in the sputum inoculated in the inulin Avery tube, the broth appears pink within from 4 to 5 hours at 37.5 C., and on longer incubation the color changes to a marked red. Incubation in the water bath, as suggested by Avery, hastens the growth of the pneumococcus, probably due to the more rapid heating of the contents of the tube to body temperature. The fact that such tubes, heated to 37.5 C. in a water bath, then quickly transferred to an ordinary incubator regulated to body temperature, yield as rapid a growth of pneumococci as do tubes incubated continuously in the water bath, would seem to bear out this contention.

When ascitic fluid is employed instead of blood, centrifugation is not necessary, prior to performing the agglutination and precipitin test. A small amount of loosely fibered cotton pushed carefully down through the broth to the bottom of the tube anchors gross particles of sputum and spontaneously agglutinated micro-organisms and yields a homogeneous supernatant bacterial suspension which can then be pipetted or even carefully poured into agglutination tubes.

#### SUMMARY OF FINDINGS BY TYPES

Of the 100 cases on which this communication is based, the following percentage of pneumococcus types was obtained:

TABLE 1  
PERCENTAGE OF PNEUMOCOCCUS TYPES

Type 1.....	33
Type 2.....	10
Type 3.....	9
Type 4.....	28
Streptococcus .....	20
Total .....	100

In each instance, the result obtained by the rapid precipitin method was checked by the Avery method. In addition, 5 of the 33 type 1 cases were checked by the mouse method and 2 by blood culture, 5 of the 10 type 2 cases by the mouse method and 1 by blood culture, 2 of the 9 type 3 by the mouse method and 1 by blood culture, 11 of the 28 type 4 by the mouse method, and 6 of the 20 streptococcus by the mouse method. In brief, in 33 % of the 100 cases, comprising all of the various types of pneumococci and the streptococcus, the mouse method or blood culture or both were employed as a check on the results obtained by the rapid precipitin method and the Avery method. The reason for the fact that only 29 cases of the series were checked by mouse inoculation was the extreme paucity of mice at the time this work was being done.

TABLE 2  
DEGREE OF INTENSITY OF THE RAPID PRECIPITIN TEST

	Type 1	Type 2	Type 3
Very marked .....	+++ 6	+++ 6	+++ 7
Moderately marked .....	+++ 11	+++ 2	+++ 2
Marked .....	++ (+) 12	++ (+) 2	++
Rather faint .....	++ 1		
Faint .....	± 2		
Negative .....	—		
Total .....	33	10	9

Of the 33 cases of type 1 infection, a positive type 1 test was obtained by the rapid precipitin method in 31; in 2 cases, the rapid precipitin method gave a negative result. In both of these cases, direct smears of the sputum revealed scattered pneumococci and large numbers of streptococci and *Micrococcus catarrhalis*. The 2 type 1 failures by the rapid method, in both of which the dextrose Avery and the inulin Avery were type 1 positive, as well as a blood culture in one case, were the only instances in which the rapid method failed. In the remaining 98 cases, the findings obtained by the rapid method agreed with the results obtained by the standard Avery or inulin Avery and the mouse method. This gives, in the series of 100 unselected cases, a 2 % failure for the rapid precipitin method. In all of the other cases in which the rapid method was negative, the Avery or mouse method failed to reveal a type 1, 2, or 3 pneumococcus and showed either a type 4 pneumococcus or a streptococcus or both. Conversely, in no instance was a positive test obtained by the rapid method which failed of confirmation by the Avery or mouse method.

The dextrose Avery method failed in 4 cases of the series, 3 of these being type 1 infections and 1 a type 2 infection. In all of the 3 type 1 failures by the standard Avery, the rapid precipitin method was type 1 positive (once ++ and twice + [ + ] ), and in each instance the mouse method revealed a type 1 pneumococcus. In the type 2 failure with the dextrose Avery, the rapid precipitin method was +.(+) positive for type 2, and the inulin Avery and mouse method were type 2 positive.

The inulin Avery method failed in one instance, a type 1 infection in which the dextrose Avery method also failed. In this case, the rapid precipitin method yielded a ++ test for type 1, and the mouse method was type 1 positive.

#### DISCUSSION

In the original communication,<sup>1</sup> reference was made to the fact that when a positive precipitin test is obtained, a clouding occurs in the fluid almost immediately on the addition of the specific antiserum. This clouding is not a homogeneous one, for when the tube is examined over a powerful electric light, more or less fine flocculi are visible within several minutes after the addition of the antiserum. This flocculation is hastened if the tube is immersed for from 10 to 20 minutes in water at 42 C. After this incubation, if the tubes are allowed to stand in the icebox for several hours, sedimentation occurs.

At present, work is being pursued on a rapid method of pneumococcus typing that will be applicable to small amounts of sputum. Such a method is especially desirable for cases of pneumonia in young children and for persons critically ill, from whom, often, only small quantities of sputum can be obtained. In two instances, in which only a small fragment of sputum was available, a micro-rapid precipitin test was used. The fragment of sputum was thoroughly emulsified on a clean glass slide in 3 drops of bile diluted with sterile salt solution to 1:10. The mixture was then carefully drawn up into a fine capillary pipet, and the tip of the pipet was sealed in a fine flame. The capillary was then immersed in water at 42 C. for 20 minutes, after which centrifugation was employed. Then 4 small drops of the clear supernatant fluid were placed in a row on a clean glass slide. To the first 3 drops were added a tiny drop of type 1, 2 and 3 pneumococcus antiserum, respectively, and thorough admixture was effected. In one instance a type 2 reaction was obtained and in the other case a type 3. In both instances, a finely flocculent clouding appeared in the respective drop

within 30 seconds, the clouding being readily apparent to the naked eye when the slide was held up to an electric light, as well as being easily visible under the low power of the microscope. In each instance, the 3 remaining drops remained clear until after about 10 minutes, when the drops had so far concentrated as to deposit a white precipitate around the edge. The type 2 micro test was subsequently confirmed by the dextrose Avery method and the mouse method, and the type 3 micro test was confirmed by the dextrose Avery method.

A somewhat more promising way appears to be the use of a 0.5 % washed agar which is allowed to solidify in a column in small, narrow agglutination tubes. In the test, 4 such tubes of agar are employed. In the first 3 tubes the top of the agar column is layered with a drop of sputum to which bile has been added. In the fourth tube, which serves as a control, the agar surface is covered with a drop of untreated sputum. The tubes are immersed in a water bath at 42 C. for 20 minutes, which is a sufficient length of time for a solution of the pneumococci by the bile and an adsorption of precipitinogen by the agar surface. The sputum is then carefully washed and pipetted off the agar column with physiologic salt solution, following which several drops of the 3 type pneumococcus antisera, respectively, are pipetted over the agar column in the respective first 3 tubes. As far as the work has progressed, it appears that when a positive test is obtained, say for a type 1 pneumococcus, the type 1 antiserum lying on the agar column, exhibits an almost instantaneous clouding if the tube is slightly shaken, whereas tubes 2 and 3 exhibit a layer of clear serum. In several instances, on incubating the tubes in a water bath at 42 C. for from 1 to 2 hours a ring test has developed below the surface of the agar column. Although by no means affording conclusive evidence, these results seem to suggest that the precipitinogen diffuses relatively rapidly into the agar, whereas the precipitin has a slower rate of diffusion. Experiments are in progress to determine the effect of layering columns of agar, into which the respective antisera have been incorporated, with bile treated sputum.

In conclusion, I may say I am by no means convinced that any conclusions can rightfully be drawn from the 4 % failure by the dextrose Avery method and the 2 % failure by the rapid precipitin method in the present series of 100 cases. It is conceivable that, in another series, the ratio of failures might be reversed. Moreover, a much larger series

than the one reported here would be required before any adequate conclusions could be drawn in regard to the comparative value of the standard dextrose Avery as compared with the inulin Avery. Theoretically and practically, so far as the present limited series goes, the modified Avery, containing inulin and an indicator, possesses certain advantages. One point of convenience lies in the fact that tubes which fail to show a red color after from 5 to 8 hours' incubation at 37.5 C. can safely be considered to be negative for pneumococci.

Finally, the rapid precipitin method has simply been advanced with the idea that, when it reveals a type 1 infection, it means a saving of from 5 to 8 hours in the administration of serum, a saving which, in certain cases, would seem to spell the difference between life and death.

#### SUMMARY

In 100 consecutive, unselected cases of pneumonia, in all but two instances, a typing of the pneumococcus has been effected within from 30 to 40 minutes by the rapid precipitin method, which was checked by the longer cultural or mouse method.

The substitution of inulin for dextrose and the addition of Andrade indicator seems to possess certain advantages over the standard Avery cultural method.

## THE OCCURRENCE OF HEMOLYTIC STREPTOCOCCI IN THE NORMAL THROAT

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The incidence of hemolytic streptococci in the throat has been studied by many workers. In normal persons the percentages as given range from 10 to 60 per cent. or higher. From the mass of literature on this point I think the impression may be gained, indeed the conclusion has been drawn by some, that this organism does not occur in the throats of some persons. Others have the impression that certain persons are definite carriers in that they constantly harbor large numbers of the organisms in the throat.

The data have been obtained in most instances by making ordinary throat swabs and then plating either by the poured plate method or by surface plate smears. These methods will determine with reasonable accuracy the incidence of hemolytic streptococci on the surface of the mucosa of the throat. It is now known, however, that hemolytic streptococci prefer to inhabit the crypts and grooves of tissues about the throat. In the crypts of the tonsils Pilot and Davis<sup>1</sup> have shown that they are found in nearly 100% of cases, and others have obtained substantially the same results. Pilot,<sup>2</sup> recently has also shown that in the folds of the adenoids these organisms are found in about 60% of cases. The surface flora and the crypt flora, therefore, are by no means alike.

The fact that in the crypts of nearly 100% of tonsils these cocci are found would clearly suggest that probably every one would have them on the mucosa at times; but I know of no definite data on this point. In order to determine whether or not this is true the following experiments were made:

Throat cultures from groups of normal adult persons were obtained. Swabs were made by firmly pressing a cotton applicator against the pharyngeal mucosa and also over the surface of the palatine tonsils when present. They were then

Received for publication June 20, 1921.

<sup>1</sup> Jour. Infect. Dis., 1919, 24, p. 386.

<sup>2</sup> Ibid., 1921, 29, p. 62.

immersed in melted blood agar and poured plates made, using proper dilutions. After 24 and 48 hours the plates were examined and suggestive colonies picked and submitted to confirmatory tests.

First the throats of a group of 15 normal persons (medical students) were examined as described; 5 or 33⅓%, gave positive results. Two weeks later a second examination was made of the same persons; 10, or 66⅔%, yielded positive cultures. Three of those yielding negative cultures were negative at the first examination of the group. Two weeks later a third examination of the same group was made and 9, or 60%, were positive. The three negatives mentioned were now positive. In the period of one month, therefore, at one time or another each of the group had hemolytic streptococci in his throat. Four gave positive results on all three examinations.

In a second group of 15 students, all different from the first group, similar tests were made but at shorter intervals. In the first test 7, or 46.6% gave positive results. After 5 days cultures were again taken from the 8 who gave negative cultures, and 3 of these were now positive and 5 still negative. One week later, cultures were again taken from the 5 who had yielded negative cultures, and 4 were positive. A culture was taken 2 weeks later; from the one remaining, who had given 3 successive negative cultures a positive culture resulted. As in the preceding series, all the persons therefore had hemolytic streptococci in their throats at some time during a relatively brief interval. Both of these series of examinations were made during Dec. and Jan., 1920-21.

Cultures were taken from a third group of 15 students in May, 1921. In the first examination 8, or 53.3%, of the 15 gave cultures of hemolytic streptococci. Ten days later 8 of the 15 gave positive cultures, but the persons were different from the first 8. Three of these who gave cultures were negative at the first examination. Eight days later these 3 negatives yielded a positive result in a third examination.

In summing up the results of these 45 examinations made 3 or more times at varying intervals, it will be seen that all at some time during an interval of about 1 month showed the presence of hemolytic streptococci in the throat. Probably they might all have been positive in a shorter time had more cultures been taken at shorter intervals. The percentages positive of the different groups varied from 33 to 66.

Nine of the 45 persons examined had had their tonsils removed months or years previously. In the first examination 3 of the 9 gave positive cultures of hemolytic streptococci; in the second examination 4 of the 9 yielded positive cultures. Of the 6 negatives in the first series, 3 were positive in the second examination. A third examination yielded 5 positives. Only one person gave negative cultures in the 3 tests. It was not possible to make further examinations on this person.

In these tests the number of streptococci in the plates were noted in relation to the other organisms. On the whole, they were few, and at times only one or two colonies appeared. Roughly, they comprised

from 1 to 10% of all the colonies that grew. One or two interesting exceptions appeared which were easily explained. One young man at work in the laboratory was feeling quite normal when the culture was taken. A cursory examination of his throat did not reveal an abnormal condition. The culture yielded an abundant almost pure growth of highly hemolytic streptococci. In 24 hours he was quite ill with fever, headache, malaise, and a red throat with fine white spots on his tonsils—a typical streptococcus sore throat. In another person the plate culture of the throat yielded between 60 and 70% of widely hemolytic streptococcus colonies. Inquiry revealed the fact that a few days before he had been ill with a cold and sore throat. The throat was fairly normal, but the tonsils were somewhat inflamed.

On the whole, the cultures from the tonsillectomized persons contained fewer hemolytic streptococci than those from persons with tonsils. There were exceptions to this, however; and in relation to incidence in this series there was little difference in the percentages in the two groups. According to previous observations of Pilot and Davis<sup>1</sup> and of others, the incidence of hemolytic streptococci in persons without tonsils is decidedly less than in those with them.

The streptococci isolated appeared to be the ordinary hemolytic variety of the human type. They were gram-positive, spherical or slightly oval cocci, some growing in short chains, others in moderately long ones. They were not encapsulated. On blood they caused a wide, clear and complete zone of hemolysis from 2 to 4 cm. wide. They grew practically not at all at room temperature, and best at 37 C. In plain broth they developed poorly but more profusely when dextrose or body fluids were added. They were no doubt the ordinary hemolytic *Streptococcus pyogenes* of the human type.

The streptococci obtained in ordinary throat swabs would appear to come from two sources. No doubt some arise from the relatively large numbers of these cocci that constantly inhabit the crypts and folds of the tonsillar and other tissues of the throat. The grooves about the teeth also not uncommonly harbor moderate numbers as Kordenat has shown. Then again a certain number appear to grow on or in the mucosa of the throat in the normal as well as in the pathologic state, as indicated by the positive cultures in the tonsillectomized persons. Presumably the latter are few, but it is difficult to



estimate exactly how numerous they may be because of the possible discharge of the same cocci from the grooves and pockets first mentioned.

#### SUMMARY

Cultures taken at short intervals sooner or later reveal the presence of hemolytic streptococci in the throats of practically all normal adult persons.

The cocci as revealed by throat swabs are not numerous; far less according to our experience than in the crypts of tonsils or adenoids.

## THE LOGARITHMIC NATURE OF THERMAL DEATH TIME CURVES

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In the article by Bigelow and Esty,<sup>1</sup> the term "thermal death point in relation to time" was used to designate the time necessary to destroy bacterial spores at a specified temperature, the kind of medium and its hydrogen-ion concentration also being given. In this article the term "thermal death time" is used to express the same idea.

In fig. 1 of the article mentioned is shown a series of curves on coordinate paper representing the thermal death times of the spores of various thermophilic bacteria at intervals of 5 C. On the opposite page are given the detailed data from which the curves were constructed. It is noted in the same article that since the curves on fig. 1 are approximately parallel, it is necessary to determine only the time required to destroy a given number of spores of an organism of this class at a given temperature in order to construct the entire curve.

The writer has since plotted the same curves on semilog paper, and the resulting straight line curves show in a much more striking manner than do the curves on coordinate paper the relations to each other of the thermal death times at different temperatures.

In table 1 of the article mentioned are shown the longest time each organism survived and the shortest time in which all spores were killed at each of several temperatures.

In chart 1 of the present article the last positive and first negative results of 6 organisms taken from the table referred to are entered on semilog paper, and the points of observation for each organism at different temperatures are connected by a straight line. Numbering the curves from left to right, the time which the organisms survived is indicated by a plus sign on the first, third and fifth curves and the shortest time required to kill them by a circle. On the second, fourth and sixth curves these times are represented, respectively, by a cross and a dot. The 6 curves represent the results from 6 typical organisms taken from the table referred to. In this way it is made clear to which curve the various observation points belong. The curves are

Received for publication June 20, 1921.

<sup>1</sup> Jour. Infec. Dis., 1920, 27, p. 602.

so drawn as to pass between the last positive and first negative in the greatest possible number of pairs of observation points with each organism. There are 30 pairs of observation points (last positive and first negative in each case) with the 6 organisms shown in chart 1.

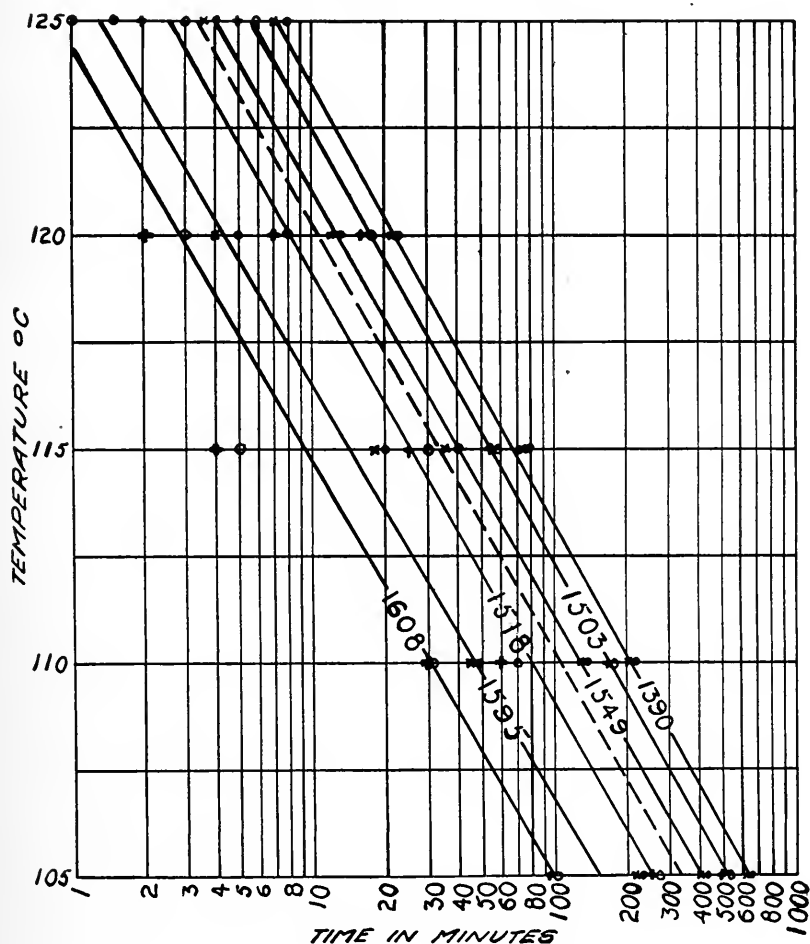


Chart 1.—Thermal death time curves.

Between 105 and 125 C. inclusive, it will be noted that the thermal death time curves pass through all but 6 of these 30 pairs of observation points. The average of the 6 curves is given as a line of bars. The data given in the table mentioned were also plotted on semilog

paper for the other 9 organisms with resulting straight thermal death time curves approximately parallel to each other and also to those shown in chart 1. These 9 curves were omitted from chart 1 in order to avoid confusion.

The similarity of these curves to one another suggested the advisability of drawing a curve which would represent the average of all 15. This would be almost parallel with each of the 15 and would serve to obliterate small errors of the individuals. The data for this

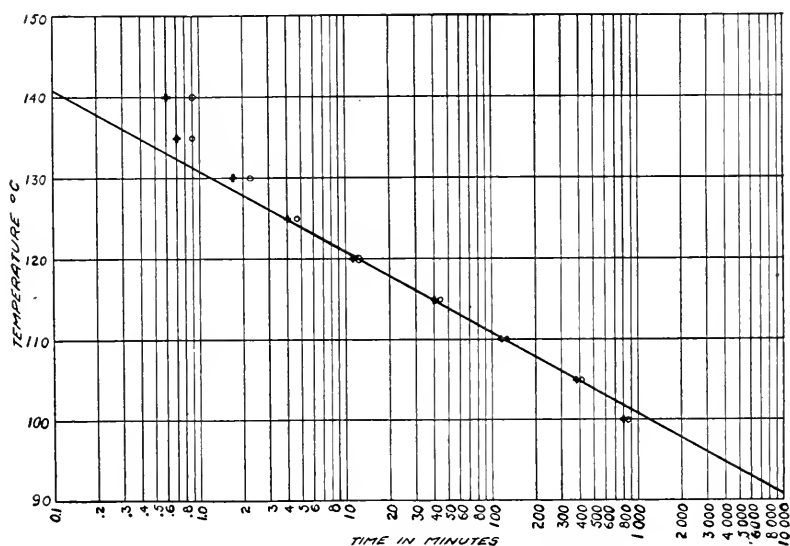


Chart 2.—Average thermal death time curve.

curve were secured by averaging separately all of the columns in the table referred to in the foregoing. These average figures are given in table 1.

TABLE 1  
AVERAGE THERMAL DEATH TIME OF 15 TYPICAL THERMOPHILES AT TEMPERATURES STATED

Temperature Degrees C.	Last Plus Reading Minutes	First Minus Reading Minutes
100	788	834
105	383	405
110	117	122
115	40	44
120	11	12
125	39	46
130	1.7	2.2
135	0.7	0.9
140	0.6	0.9

The data shown in this table are arranged on semilog paper in chart 2, the last positive result being represented by a plus and the first negative result by a circle.

It will be noted that the curve is a straight line and passes between all positive and negative observation points from 105 to 125 C. inclusive. Between these temperatures, therefore, the thermal death time curves of these 15 typical thermophilic bacteria are logarithmic curves and can obviously be drawn more accurately on semilog paper than on coordinate paper.

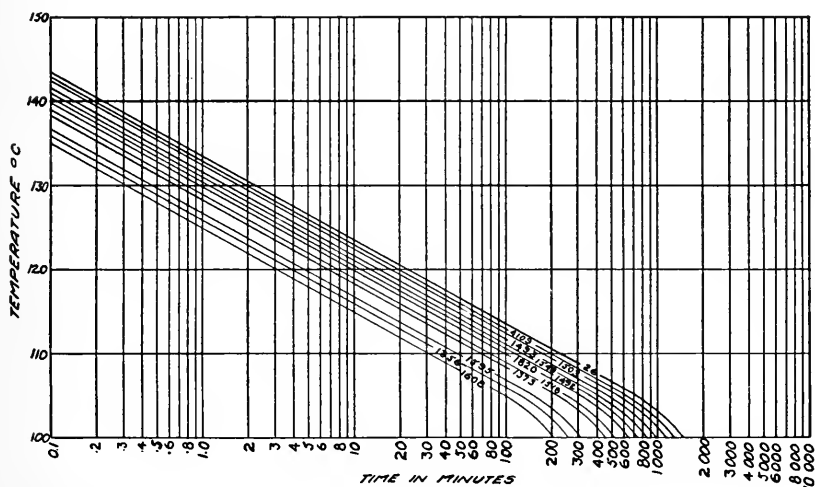


Chart 3.—Thermal death time curves of typical thermophilic organisms PH 6.

The typical thermal death time curve shown in chart 2 does not intercept the observation points above or below the temperatures noted. At higher temperatures this difference is probably explained in part by the heat penetration of the small tube in which the thermal death time was determined. The tube is so small that the error of heat penetration is not great, but the time required to destroy the spores at temperatures above 125 C. is so short that the retarding influence of heat penetration is doubtless partly responsible for the deflection of the observation points from a straight line.

In chart 3 the thermal death time curves on semilog paper are given for 12 of the 15 organisms in question. The curves for the other three organisms would have practically coincided with three of those shown. These are drawn in such a way as to coincide as nearly as



minutes for their destruction at 130 C. and the time indicated by the curves in chart 3 is noticeably less than the observed time.

The 15 organisms under discussion resemble each other in some respects but exhibit many dissimilarities. All are facultative anaerobes and none are gas-formers. The majority of them produce acid. One of them produces a bitter substance which has not been identified and another secretes an enxyme which hydrolizes starch to maltose. There are other marked points of dissimilarity which will be discussed by the associates of the writer in subsequent papers. Their dissimilarity is merely mentioned here as that fact considered in connection with the parallelism of the thermal death time curves suggests a general law which may be of value from a biologic standpoint and which is a great convenience in thermal death time work.

Since the curves are straight parallel lines when drawn on semilog paper, a type curve having the proper slope may be drawn on a blank sheet of the paper, as shown in chart 2. The time necessary to destroy a certain number of spores of a given bacterium may be determined and the last positive and first negative results entered in their appropriate positions on the same paper. Lines drawn through these points parallel to the type curve fix quite definitely the thermal death time curve of the organism between the temperatures of 105 and 125 C. These lines may be confirmed at other temperatures much more readily than by any other procedure, and a much smaller number of observations is required to draw an accurate curve than would otherwise be necessary.

It becomes a matter of interest to determine how generally this method of procedure may be applied to thermal death time work. The change in the direction of the curve below 105 C. suggests the possibility that the deflection may be due to some biologic change in the spores resulting from a closer approach to the temperatures at which they will germinate and the vegetative cells will grow. The maximum temperature of growth of the spores in question has not been determined in all cases but in some cases it is above 75 C. It would be interesting to extend these curves down to the maximum temperature of growth of the bacteria, but that procedure is rendered extremely difficult by the long time that would be required to destroy the spores at the lower temperatures.

The thermal death time curves shown in chart 3 are also shown in chart 4 on coordinate paper. In general, the latter do not differ greatly from the same curves shown in the article by Bigelow and

Esty,<sup>1</sup> although there is considerable difference in detail, especially at the lower temperatures. The advantage of using the straight line in connection with semilog paper and the advantage which semilog paper affords of comparing with other organisms suggests in the writer's opinion that where differences occur the curves, as shown in chart 4, are more representative of the thermal death time of the organisms than those shown in the article referred to.

It is of interest to note also whether the method of procedure described in the foregoing is applicable to nonspore-bearing bacteria.

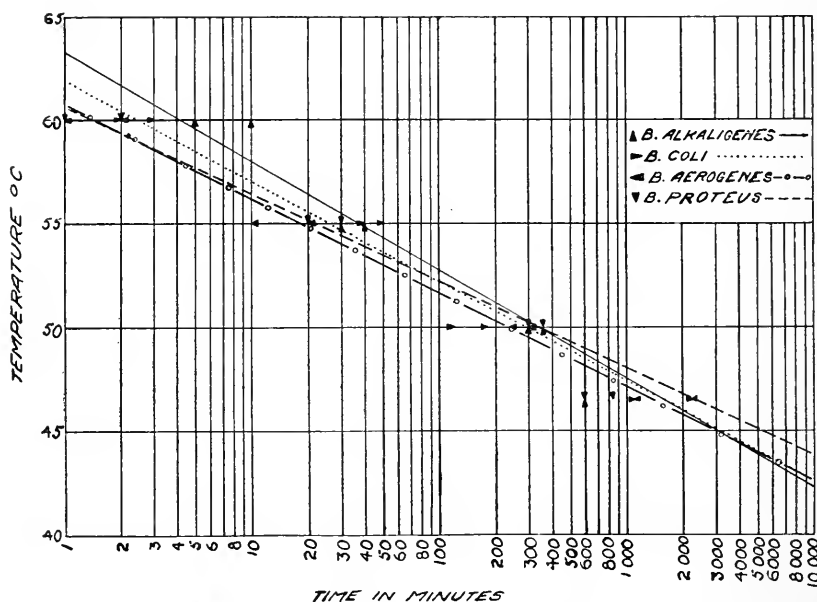


Chart 5.—Thermal death time curves of four non-spore-bearing organisms.

Unfortunately, work of this kind has not been carefully controlled and the literature does not appear to afford enough observation points to permit the plotting of the data recorded.

Four strains of nonspore-bearing bacteria were obtained through the kindness of Mr. L. A. Rogers, and their thermal death times were determined by the method described in the paper mentioned. The results are given in chart 5.

The points of observation are indicated by solid triangles as shown in the legend on chart 5. It was impossible to differentiate between the last positive and first negative results for each organism because



of the number of characters necessary to give the data. This can be done readily by inspection. The last positive and first negative results are given in all cases except for *B. alkaligines* at 46.5 C., at which temperature no positive result was obtained for that organism. The curves shown in chart 5 do not intercept the points of observation as closely as in the case of the thermophiles thus far studied. This may be due, in part, to lack of sufficient data. From the nature of the case it was impossible to operate at temperatures widely different from each other. If this could have been done, more accurate curves

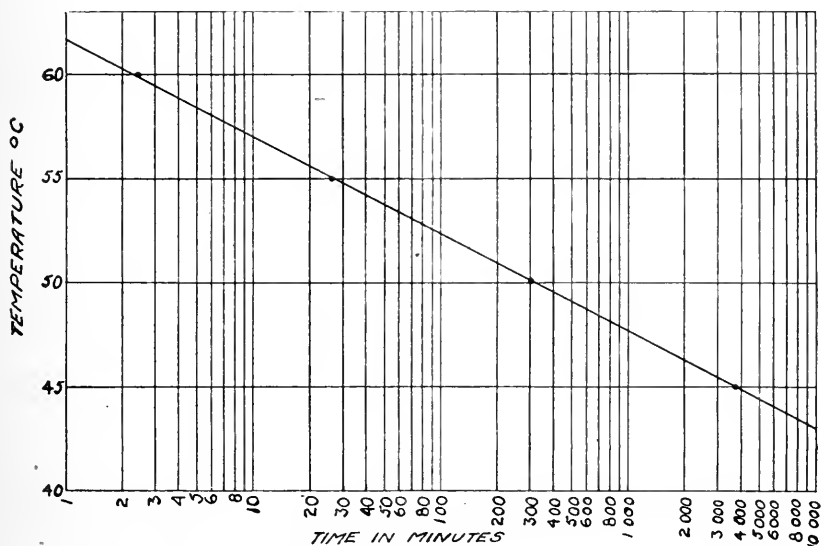


Chart 6.—Average thermal death time curve of four non-spore-bearing organisms.

might have been obtained. The results are sufficiently consistent, however, to suggest that thermal death time curves of nonspore-bearing bacteria are logarithmic.

These curves are not as nearly parallel as those of the various thermophilic spores, but they approach parallelism sufficiently closely to be convenient in thermal death time study and possibly to be of value in the study of other biologic relations in micro-organisms. The curves in question represent only a few determinations and should be confirmed before being definitely accepted. They are given here more to illustrate the general question of the nature of the curves than as a record of thermal death time.

The mathematical average of the four curves shown in chart 5 was calculated at four temperatures, and the results are indicated in chart 6 by dots connected by a straight line which is therefore the average curve of the four thermal death time curves shown in chart 5. It will be noted that the slope of this curve is only about one half that of the curves shown in charts 1 to 3 inclusive for thermophilic organisms. It is probable that this curve will coincide sufficiently closely in slope with the curves of many nonspore-bearing bacteria to make it of value as a guide in thermal death time work. It is hoped that other workers in this field will apply the method in order that the extent of its application may be determined. It is suggested that the thermal death time curve of nonspore-bearing bacteria which will not grow between the temperatures of 45 and 60 C. will probably be approximately parallel to the curve shown in chart 6; if not, the direction of the semilog thermal death time curves may afford an additional means of distinguishing species or classes of bacteria.

The foregoing data were secured by heating spores in juice expressed from canned corn with a  $p_H$  value of 6.1. Somewhat different curves are secured at other  $p_H$  values.

## THE BACTERIOLOGY OF PERIDONTAL TISSUES RADIOGRAPHICALLY SUGGESTING INFECTION \*

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The medical and dental literature for a number of years has been filled with reports and bacteriologic data concerning chronic alveolar infections. A critical review of the wealth of material reveals one outstanding feature, namely, that no single report is based on a uniform and reliable bacteriologic technic. This applies especially to the method of obtaining cultures. Moreover, the reports indicate a lack of standardized operative technic and some form of statement which would prove that carefully conceived series of controls have been carried out. It is not unlikely that the cultures obtained and described at some length in certain publications were merely contaminations from the oral flora.

For this reason I have undertaken to investigate the bacteriology of chronic alveolar infections as evidenced by shadow changes in the radiogram; to determine the relationship of the cultures obtained to the bacteria of the oral cavity; to ascertain, if possible, the relationship of chronic alveolar infections to systemic disease.

*Method of Study.*—In a previous article I<sup>1</sup> reported a method of sterilizing the oral mucosa which proved satisfactory not only from the laboratory point of view, but also from the clinical aspect. The cultures were made from tissues dissected from jaws showing definite radiographic changes. The material was obtained as follows: The teeth were first brushed with an alkaline tooth powder and then the mouth was rinsed with an alkaline lotion. The alkaline reaction of the tooth preparation intensifies the bacteriostatic action of the dye. The gums and mucous membrane of the buccal surfaces were painted with a solution containing 1% each of brilliant green and crystal violet, dissolved in 50% alcohol. Churchman has shown that it is possible to inhibit the growth of gram-positive cocci in dilution of gentian violet up to 1:5,000,000 in alkaline solution. Hence, in every instance care was taken to avoid a transfer of dye into the culture medium with

Received for publication June 27, 1921.

\* Aided by a grant from the San Francisco Society for Dental Research.

<sup>1</sup> Jour. Dental Research, 1920, 2, p. 21.

the swab. The solution was applied after packing the area off with sterile gauze or cotton rolls. The dye was allowed to penetrate the tissue two and one half minutes. The gum was then incised and a flap of gum and periostaeum laid back with a periosteal elevator. The buccal plate was then entered with a chisel and with a clean chisel or rongeur forceps a piece of tissue at least 4-5 mm. in diameter was removed aseptically. This was then placed in a culture tube containing 1% glucose-veal-broth and taken immediately to the laboratory. The tissue was macerated with sterile sand broth according to the method described by Rosenow. The tissue pulp was enriched in glucose-blood agar and in glucose-veal-broth  $P_H$  7.4, to which were added 10% defibrinated blood. The tube in which the tissue was taken to the laboratory was also incubated as a control.

TABLE 1  
GENERAL SUMMARY

Type of Local Lesion	Hemolytic Strepto- cocci		Non- Hemo- lytic Strepto- cocci	Staph- ylo- coccus, Pure	Strepto- cocci With Staphyl- ococcus	Other Organ- isms	Sterile	Total Number Culti- vated	Age of Patients, Years
	Pure	Mixed							
Sclerosis.....	..	..	2	..	1	..	..	3	25-43
Neerosis.....	1	1	8	5	14	..	3	32	32-65
Granuloma....	..	1	8	2	4	..	..	15	18-71
Cyst.....	..	..	..	..	3	2 unclass- ified ana- erobes	4	9	38-64
Pericementitis*	..	..	4	1	5	..	2	12	24-62
Total.....	1	2	22	8	27	2	9	71	

\* I have used the term *pericementitis* to cover the terms *alveolitis* and *periodontitis* as used by McCormack.

Swabs were made from the surface of the gum after the application of the dye just before the surface was incised. They were washed out in glucose-blood-agar and the latter poured into plates. The washed swab was placed in glucose-veal-broth and incubated as usual. Invariably these control cultures were found to be sterile after 48 hours of incubation. Occasionally control cultures were also made of areas appearing normal in the radiogram.

The bacteria isolated by the method described in the foregoing were identified, and, as far as the streptococci were concerned, they were classified according to the method of Holman.<sup>2</sup> Later, with the appearance of Brown's monograph,<sup>3</sup> they were differentiated first by

<sup>2</sup> Jour. Med. Research, 1916, 24, p. 377.

<sup>3</sup> Monographs, Rockefeller Institute for Medical Research, 1919.

hemolysis and then with 1% mannite, lactose, salicin, raffinose, inulin and sucrose, in beef serum with Andrade's indicator.

*Results.*—The cultures were obtained from jaws which demonstrated radiographically certain distinct changes. These have been illustrated by McCormack,<sup>4</sup> and in this paper the tentative classification given by him has been followed. It should be said in this connection that the bacteriologic study was primarily undertaken to elucidate definitely the meaning of some of the radiographic changes demonstrated and discussed by McCormack. It is quite evident that the bacteriologic data failed on some occasions to confirm the original radiographic interpretation.

TABLE 2  
CASES WITH SYSTEMIC DISTURBANCES

Case	Age	Disease	Number of Teeth Extracted	Postoperative Condition	Cultures Obtained
1	60	Atrophic arthritis	1	Not improved after 24 months	Hemolytic streptococcus
2	62	Neuritis	2	Improved after 25 months	Staph. albus Strep. salivarius
3	27	Hypertrophic arthritis	5	Very slightly improved after 22 months	Staph. albus Strep. nonhemolyticus I
4	60	Arthritis deformans	6	Not improved after 22 months	Staph. albus
5	33	Neuritis	10	Very slightly improved after 23 months	Streptococcus alpha 2.1 Streptococcus beta 8.5
6	40	Occipital headaches	11	Not improved after 24 months	Staph. albus Strep. salivarius
7	62	Atrophic arthritis	2	Not improved after 20 months	Staph. albus Streptococcus alpha 1.1 Streptococcus beta 2.1
8	45	"Run-down"	2	Improved after 23 months	Staph. albus Streptococcus alpha 4.5
9	32	Acute arthritis	7	Worse after 24 months	Staph. albus Streptococcus alpha 2.1
10	37	Hypertrophic arthritis	2	Not improved after 16 months	Staph. albus Streptococcus alpha 1.1

Table 1 shows the results of our bacteriologic study classified according to the radiographic and pathologic picture. In all there were 71 cultures taken. Of the total number of cultures obtained, we find only 3 hemolytic streptococci and of these 2 were found in large necrotic areas whereas 1 was isolated from a granuloma. At the same time we note that there are in all 22 pure cultures of nonhemolytic streptococci, and 8 pure cultures of staphylococci. A large number of our cultures showed that the infection was a mixed one and that often there were 2 or more different strains of streptococci in the material used for culture.

<sup>4</sup> Jour. Dental Research, 1920, 2, p. 467.

Two of the organisms we obtained from a large cyst were anaerobes and further classification of these bacteria was not possible. Originally the organisms were obligatory anaerobes but with replanting they became facultative anaerobes.

Nine specimens of tissue taken for culture remained sterile, 3 of these coming from necrotic areas, 4 from cysts and 2 from areas of pericementitis.

In addition to this group, 3 other specimens showing apparently normal bone regeneration following extraction were cultivated. All gave sterile cultures.

There were other cases besides those operated on for the removal of local changes, which deserve more detailed analysis (table 2).

TABLE 3  
DATA CLASSIFIED ACCORDING TO THE TOOTH INVOLVED IN THE INFECTION

Tooth		Strepto- coccus	Staphylo- coccus	Mixed Infection	Sterile	Total
Bicuspid.....	Upper	6	4	8	3	21
	Lower	2	2	2	1	7
First molar.....	Upper	4	..	4	1	9
	Lower	6	..	2	..	8
Second molar.....	Upper	..	..	4	2	6
	Lower	1	1	..	..	2
Cuspid.....	Upper	1	1	1	1	4
	Lower	..	..	..	..	0
Incisor.....	Upper	..	1	1	..	2
	Lower	..	..	..	..	0
Third molar.....	Upper	1	..	..	..	1
	Lower	..	..	1	1	2

The cases shown in table 2 are those that had been thoroughly examined by competent physicians, and primary causes as well as sources of infection other than dental were eliminated. After removal of the dental infection, a sufficient period of time was given in each case for the symptoms to improve or the changes to decrease. The shortest period of time allotted was 16 months and the longest 25 months.

Only two patients improved definitely, whereas two improved slightly, one was made worse and the rest were unaffected. An examination of the cultures from these cases showed that there were three hemolytic streptococci, one in pure culture and two mixed with other organisms; one pure culture of staphylococcus was obtained in a particularly bad case of arthritis deformans which was not improved; the predominating type of organism here also is the nonhemolytic streptococcus.

Table 3 shows that the upper bicuspid area gave over 33% of the cultures and that the upper and lower bicuspid areas together gave 45% of the total.

The reason for this predominance of bicuspid involvement is perhaps evident when one considers the great variation in the position, size and shape of the pulp chamber and the pulp canal of the bicuspids, especially the superior. Hopewell-Smith<sup>5</sup> and Marshall<sup>6</sup> give the variation from the normal at about 50% in regard to the upper bicuspid, whereas the variation from the normal of the other teeth is much less.

There appears to be no existing relationship between the type of infecting organism and the tooth concerned.

#### COMMENTS

This investigation shows that 9 cases of 71 gave sterile cultures. Three of these were from large necrotic areas, four from cysts of the jaw, and two from alveoli which showed definite thickening in the radiogram and in the pathologic examination. This definitely proves that cultures can be taken in the buccal cavity uncontaminated by the oral flora. It also shows that all changes in the jaw as evidenced by radiographic findings are not infected. Thoma<sup>7</sup> "believes that all teeth which give roentgen evidence of bone involvement are infected." Our evidence certainly does not bear out this statement. For over 10% of our cases proved sterile although there certainly was radiographic evidence of apparent bony change about the teeth in question.

The examination of the cultures we obtained shows that they correspond to a great extent to the cultures of organisms of the buccal flora (Meyer<sup>8</sup>). Arnold<sup>9</sup> also finds that the "hemolytic and nonhemolytic streptococci found in normal and pathological throats were of the same varieties, when classified according to Holman's sugar fermentation tests."

Under ordinary conditions these may be innocuous, but, due either to a temporary increase in virulence of the organism or to a local lowering of resistance, the bacteria may have gained a foothold on the tissue and there produced their local effects. The lesions produced by these organisms, when situated deep in the jaw, appear to clear up spontaneously in a few cases only, but appear to remain locally until removed by the dentist.

<sup>5</sup> American Textbook of Operative Dentistry, 1920, p. 83.

<sup>6</sup> Operative Dentistry, 1921, p. 14.

<sup>7</sup> Boston Med. and Surg. Jour., 1921, 184, p. 434.

<sup>8</sup> Jour. Nat. Dental Society, 1917, 4, p. 966.

<sup>9</sup> Jour. Lab. and Clin. Med., 1921, 6, p. 312.

That the majority of the organisms situated here are capable of producing pathologic processes elsewhere in the body appears from our data at least to be improbable. However, further investigation is being carried out to ascertain this point.

The cases showing hemolytic streptococci are three in number, one in pure culture and two in mixed growths. The virulence of hemolytic strains of streptococci have long been said by a few to be greater than the majority of the other types of this organism. In examining table 2, which contains the group of cases showing the so-called systemic disturbances, we find that the predominating type of streptococcus is the nonhemolytic, and in 80% of these cases, it was found in mixed culture, either with another type of streptococcus or with staphylococci. In one of these cases a pure culture of staphylococcus was obtained. It is true, however, that all of the hemolytic cocci we obtained were found in this group of cultures. It is not unlikely that a larger series of cultures may show different results.

The eradication of these local dental infections, if they are related to processes elsewhere in the body, should produce some indication of reparatory change in the latter, provided, of course, that sufficient time be given and the damage done is not irreparable. Of the three cases furnishing hemolytic streptococci in their dental tissues, only one showed a slight improvement in the general condition.

These facts may possibly be explained as follows: The patients had other foci from which bacteria may have originated and which act as a source from which reinfection may take place. There are permanent changes in the body and even with the removal of the cause, the damage already done cannot be repaired. The dental focus may have no relation to the systemic disorders since time enough was allowed in each of the instances cited to allow the condition to improve following the removal of the infection by radical measures. However, careful physical examinations were made in each of the cases in table 2. Special care was exercised to search for foci of infection other than dental and to rule out any other pathologic condition likely to produce the condition under observation. In each of these instances the only definitely demonstrable anatomic changes which remained were in the mandible or maxilla.

It must be remembered that extensive hypertrophic changes in the osseous system cannot be greatly affected by the removal of the cause. The progress of the infection may be altered to some extent but without effect on the existing process.



From our series it appears that there is no definite relationship between dental infections and the symptoms complained of by the patient. The histories are not as complete as one desires and a larger series of cases is therefore being investigated along this particular line.

#### CONCLUSION

In a series of 71 cultures no type of organism was found to be characteristic for any radiographic change which had taken place in the maxilla or mandible. Contrary to expectation, it was noted that 10% of the cases were sterile. It is not possible to ascertain from the radiogram the existence of an infection in any case. The bacteria isolated from areas showing radiographic change correspond to a great extent to those found in the oral cavity. It is not possible to state from the results that there is any definite relationship existing between dental infection and systemic disorder in more than a small percentage of suspected cases.

# INCIDENCE OF TUBERCULOSIS IN THE VARIOUS ORGANS OF THE PIGEON

OSCAR RIDDLE

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It is known that tuberculosis of the common fowl is much more frequently found in the liver and spleen than in other organs; that this disease is produced by the avian form of the tubercle bacillus; that other birds and some mammals are susceptible to experimental inoculation with the avian bacillus, and that the pigeon and guinea-pig are probably less easily inoculated than are the fowl and rabbit.

In the course of investigations on sex in doves and pigeons during the past ten years, it has been necessary to make rather careful necropsies on several thousands of these animals. During the last seven years of this period data concerning tuberculosis of each bird were so recorded as to designate the organ which from macroscopic examination seemed most extensively affected; other affected organs were given rank in the order of the extent to which they were invaded by tubercles. In making such a classification there is undoubtedly some opportunity for error and still another factor is introduced by the circumstance that only about six-sevenths of the necropsies were made by the same observer; during one year of the seven-year period all of the necropsies were made by Dr. E. H. Behre. It is thought, nevertheless, that our records afford an approximately consistent and correct description of the facts.

A summary of the classified data thus obtained has recently been made. There is, perhaps, some reason for believing that this summary may be of interest to others than ourselves. Nearly similar data for birds are apparently available only for the common fowl, and in view of a certain amount of knowledge concerning the localization or distribution of phagocytic activity in the organs of the pigeon, the results set forth here may have some bearing on the general subject of tuberculous infection and on the subject of phagocytosis.

Apparently no data are available which show the relative extent to which the various tuberculous organs of any bird were invaded by tubercles. Data for the number of times tuberculous infection was found in each of the organs of the fowl have, however, been supplied by Vosgien<sup>1</sup> and by Raymond and Crétien.<sup>2</sup> Necropsies made by Vosgien on 145 fowls condemned in a public

Received for publication July 1, 1921.

<sup>1</sup> *Hygiene de la Viande*, 1912, 6, p. 207.

<sup>2</sup> *Ibid.*, 1912, 6, p. 211.

market in Paris showed the following distribution of tuberculosis in the various organs: liver, 143 cases; spleen, 135; lymph glands of neck, 102; lungs, 61; bones and joints, 46; peritoneum, 13; kidneys, 9; ovaries, 6. The organ was considered tuberculous when enlarged and provided with yellowish caseous nodules. Several of these nodules were stained and tested by the method of Ziehl, and it is stated that tubercle bacilli were invariably found.

Raymond and Crétien continued the work of Vosgien and, though they too used fowls condemned later in the same market, obtained somewhat different results. They state that in a tuberculous fowl "the liver and spleen are always infected with tubercle bacilli." It is not clear whether they performed necropsy examinations on 766 or only one-half this number of fowls since they merely give the number of cases of tuberculous "liver and spleen" as 766 cases. Other organs follow in order: lymph glands of neck, 164; bones and joints of hip and shoulder, 79; lungs, 23; gastro-intestinal canal, 17; kidneys, 12; ovary, 3; thyroid, 2. It seems highly probable that Vosgien studied more advanced cases of infection—cases more nearly comparable with our own—and that Raymond and Crétien made use of birds in a much less advanced stage of infection. The report unfortunately leaves this a matter of conjecture. In making tests on the nature of the infections these workers made peritoneal inoculations of rabbits with an emulsion of the caseous exudate of the hip joint of the fowl. Three months later all rabbits are stated to have exhibited tuberculosis of the mammalian type. In these cases "the liver and spleen were much enlarged and bore bacilli but no tubercles." A fowl inoculated in the axillary vein with the exudate from an infected articulation was found emaciated at the end of 2 months and was then killed. The liver and spleen were considerably enlarged and contained many small grayish nodules in which the bacilli were numerous. Other investigators have successfully inoculated fowls, ducks, geese, pigeons, etc., with this bacillus.

These citations have been rather fully considered, not only because they seem to include practically all that has been learned of the relative incidence of tuberculosis in the various organs of birds, but because the bacteriologic identification of the disease has been attempted by these workers while it has been quite neglected by ourselves. If, however, the identification of the nature of this infection is established in the case of the fowl, there can be little doubt that at least nearly all the data presented here are likewise from true cases of avian tuberculosis. We had learned to recognize tuberculosis in the fowl prior to our more extensive experience with pigeons; the similarities of the disease in the two forms are too pronounced to be easily mistaken.

One statement concerning the external symptoms of the disease as it presents itself in the pigeon may be added: Emaciation is not evident; assuredly it is not prominent in all cases of advanced tuberculosis; and we have listed as unquestionable some cases of tuberculosis in ring-doves with abnormally high body weight and in which the large pectoral muscles were of normal or nearly normal size. In these cases the excess weight was largely accounted for by the enlargement of the

liver and spleen; in some instances the latter organ was enlarged from 0.1 gm. to 11.0 gm. On the other hand, it is our opinion that advanced tuberculosis in the pigeon is always accompanied by resorption in the ovary of all ova of more than 1.0 mm. diameter, and by extreme atrophy of the testes.<sup>3</sup> Rarely does death from another cause bring about so profound a change in these organs.

Our data were obtained from 940 tuberculous Columbidae which we have divided according to kind or origin into 5 groups. Nearly three fourths of these birds were adults dead of disease; about 215 (mostly belonging to group 3) were older than 3 months and were either killed for necropsy while supposedly healthy or were killed by accident. The method used in obtaining these data do not permit a numerical statement of the proportion of all doves which are attacked or killed by tuberculosis; this proportion, however, is high among those individuals, in most or all kinds of pigeons, which are otherwise permitted to live their full span of life. The intestines of many of the tuberculous birds were found to be also badly infested with *Ascaridia*. Other diseases were of course also occasionally present and in some instances these were accompanied by tuberculosis. The head and neck regions were not included in the necropsies except as these plainly displayed bone or joint tuberculosis. The lymphatic glands, thyroid, crop, etc., are omitted in our necropsies and tabulations.

Table 1 gives the details of data obtained for the 5 groups. The data indicate that in pigeons the spleen and liver are each about twice as often the seat of tubercle formation as are the lungs, and that the latter are about 3 times as often infected as the mesenteries; the joints, ovaries, abdominal wall and intestine follow next in order; in still other organs the tubercles are rarely but probably not equally rarely found. In all groups except the common pigeons the spleen is shown to be the most frequently infected organ. The liver ranks second in 4 cases and first in the group of common pigeons. The lungs rank third in 4 cases and fourth in 1. It is again among the common pigeons that this irregularity appears—the joints here being more often infected than the lungs. Elsewhere the joints rank fourth in two groups and fifth and seventh in the other 2 groups. The mesenteries were more often infected than the joints in only 2 of the 5 groups, but the sum of all groups shows a considerable excess of infected mesenteries. The ovary ranks sixth, the intestine seventh and the abdominal wall eighth. Other organs are infected with incon-

<sup>3</sup> Riddle, O.: *Anat. Rec.*, 1918, 14, p. 283.

siderable frequency. Our data indicate—though the numbers of recognizable infections are here so few as to be of rather uncertain value—that these organs take the following order: testes, kidney, oviduct, pericardium, suprarenals, pancreas, heart, gizzard and cloaca.

The details of the tabulated data—omitted here to economize space—make it clear that in all groups of birds the particular organ which was most often infected was at the same time the most affected organ in the greatest number of cases. Perhaps this circumstance may be taken as an indication that the most frequently infected organs are themselves the seats of the primary infection.

The numbers are large enough and the agreement of the 5 groups is close enough to make it quite clear that the spleen, liver and lungs are most extensively, as well as most often, infected; and that these organs are infected in essentially the order named, with the exception that in common pigeons the liver is probably more frequently the seat of attack than is the spleen. It is highly probable that the mesenteries, joints and intestines follow, but not necessarily in this order, in all groups of pigeons. For the other organs it is certain only that the number of infections is small and markedly less than in any of the organs mentioned in the foregoing in the kinds of pigeons which we have examined. In the case of some of these infrequently infected organs, such as the suprarenal and testis, it is quite possible that the infection originated external to them and merely enclosed or surrounded them.

It is of interest that the data afford good evidence that the ovary is more often the seat of infection than is the testis. The present data were collected incidental to investigations on sex; the gonads were therefore examined with particular care in the necropsy examination of every bird—not only of the 940 individuals showing tuberculosis, but in many thousands of healthy pigeons whose ages ranged from embryos to adults. Other cases than those listed of advanced tuberculosis in these organs were almost certainly not present. Very few, however, of the cases of tuberculous gonads listed were examined microscopically (none were stained for bacilli) and it is possible that some or several of the cases listed were really tumors, not tuberculous. Proper classification of disease in these organs by macroscopic observation is made questionable and especially difficult by the circumstance that the size of the testes is greatly diminished, often to one-fiftieth of their full and functional size, in the case of males whose other organs showed advanced tuberculosis.<sup>3</sup> In the ovary there is little or no corre-

sponding change of size when the spleen, liver, lungs, etc., are highly tuberculous; but, in these ovaries all ova of more than 1.0 mm. atrophy and no younger ova advance beyond this size.

It is notable that among the common pigeons the testis is as often infected as is the ovary. Indeed, tuberculous testes were almost wholly absent in other groups. Five of the total of 7 cases of tuberculous testis were supplied by only 57 tuberculous male common pigeons while the 317 male ring-doves of group 3 yielded no cases of tuberculous testis; the 306 females of the latter group supply 14 cases of such ovaries. Here once more the differences in the incidence of tuberculosis in common pigeons and in other pigeons is marked. The rank or order of infection assigned in the tables to the sexual organs takes account of the fact that the numbers of males and females in the 5 groups were: group 1, 57 males and 63 females; group 2, 35 males and 22 females; group 3, 317 males and 306 females; group 4, 38 males and 27 females; group 5, 39 males and 18 females; a total of 486 males and 436 females. The few remaining birds were either hermaphroditic or for some other reason their sex could not be ascertained. A total of 18 of the 940 birds are unclassified as to sex and of this number 17 are from the 2 groups of generic hybrids.

#### DISCUSSION

It has been observed that the spleens and livers include more than two thirds of the instances of tubercle-bearing organs.

Kyes<sup>4</sup> found that the endothelial cells (hemophages, Kupffer cells) of precisely these two organs in the pigeon remove nearly all intravenously injected pneumococci from the blood stream. Kyes says, p. 282:

"At all intervals between 10 minutes and 72 hours the liver contained many more pneumococci than did any other organ. The spleen ranked next. . . . Cell for cell, the hemophages of the spleens contained as many pneumococci as did those of the livers. The absolute numbers of hemophages was, however, as in normal pigeons, distinctly smaller."

Here also the lungs were found to rank third in order of importance and in "other organs the numbers were relatively inconsiderable"—microscopic examination failing entirely to find the organisms in breast muscle and genitalia.

In other animals some interesting parallels and contrasts are known. In rabbits Hopkins and Parker<sup>5</sup> found 10 minutes after intravenous injection of streptococci the following distribution of the organisms: spleen, 120,000; liver, 104,000; lung, 41,000; psoas, 1,500; kidney, none. These figures for this property of the organs of the rabbit may be seen by reference to our summary table to afford an odd and nearly perfect parallel—even on a percentage basis—of the foregoing to the relative incidence of tuberculosis in the organs of most

<sup>4</sup> Jour. Infect. Dis., 1916, 18, p. 277.

<sup>5</sup> Jour. Exper. Med., 1918, 27, p. 1.

kinds of doves other than common pigeons. In cats, however, Hopkins and Parker found quite a reverse order of distribution of the organisms in the three organs chiefly involved, namely: spleen, 18,000; liver, 34,000; lungs, 315,000.

In their recent study of the distribution in the cat of finely divided manganese dioxide after intravenous injection, Drinker and Shaw<sup>6</sup> were able, when a particular concentration was used, to recover 90% of this material in the lungs, liver and spleen, and in the following proportions: lungs, 47%; liver, 38.4%; spleen, 4.3%. It is thus clear that the order of distribution of the manganese dioxide is similar to the distribution of the streptococci found by Hopkins and Parker for the organs of the same animal. Drinker and Shaw state, p. 91:

"Although we cannot make a direct and final comparison between the intravenous bacterial injections and intravenous injections of nonliving material such as the manganese dioxide used in this study, it seems established that in as far as the immediate removal from the blood stream is concerned the two types of injection are treated similarly by the animal."

The studies last mentioned are cited because of the nature of the analogy involved and even more because in a further publication by Lund, Shaw and Drinker<sup>7</sup> this study has been extended to other animals, including one bird—the common fowl. In this bird the manganese dioxide was recovered as follows: from the liver, 84.0%; spleen, 1.7%; lungs, 0.2%; all other organs, 14.1%. From these and other figures tabulated by these workers it is fairly clear that this nonliving substance is distributed in the organs of the fowl in nearly the same relative order as those of the rabbit and quite differently from the order found for the cat. And, as noted in the foregoing, both the relative order and percentage distribution of the streptococci in the rabbit is closely parallel to the frequency of tuberculous infection in fowls and pigeons. The relative order of distribution of the manganese in the organs of the fowl is also rather similar to the incidence of tuberculous infection in the pigeon, although the percentages involved are apparently quite different.

The correspondence to which attention has just been drawn may afford a slight basis for the suggestion that the various organs of the pigeon become the seat of tuberculous infection with a frequency which is proportional to the number of infecting organisms which they remove from the blood stream. Stating the point otherwise, we may say that earlier work has demonstrated the phagocytic capacities of the various organs of the pigeon to be of about the same relative order as we now find these organs infected with tuberculosis.

It remains to mention two further incidental points of possible interest. The data may be of slight value in a consideration of the question whether tropical species brought to or reared in New York are more or less susceptible to pulmonary tuberculous infection than are species native to temperate climates. The results fail to indicate any such influence. It should be stated, however, that all of these birds

<sup>6</sup> Jour. Exper. Med., 1921, 33, p. 77.

<sup>7</sup> Jour. Exper. Med., 1921, 33, p. 231.

were protected from extreme cold in heated buildings. It is obvious, moreover, that the birds which supply the present data may have been affected or predisposed, or certain organs thrown under special strain, by the general nutritive or other conditions provided by us and that this may have affected the data obtained for all of the groups. Concerning this it can be said that, because of the nature of the work being

TABLE 1  
CONDENSED SUMMARY ON THE INCIDENCE OF TUBERCULOSIS IN PARTICULAR ORGANS IN THE PIGEON. NUMBER OF PLAIN CASES OF TUBERCULOSIS (= A) AND OF PROBABLE EARLY STAGES (= B) \*

	Group I 120 Birds		Group II 57 Birds		Group III 624 Birds		Group IV 76 Birds		Group V 63 Birds		Total 940 Birds	
	A	B	A	B	A	B	A	B	A	B	A	B
Spleen.....	36	4	34	2	465	80	54	11	29	7	618	104
Liver.....	51	23	27	12	394	102	38	20	27	12	537	119
Lungs—Both....	9	0	17	0	75	15	9	1	12	0	122	16
Right.....	10	0	5	0	73	1	10	0	10	0	108	1
Left.....	12	0	5	0	40	0	6	0	5	0	68	0
Total....	31	0	27	0	188	21†	25	1	27	0	298	22
Mesentery.....	27	0	1	0	64	3	10	0	4	0	106	3
Joints.....	38	0	6	1	18	1	5	0	7	0	74	2
Intestines.....	6	0	3	0	29	6	4	1	...	...	42	7
Abdominal wall..	12	1	4	0	21	0	5	0	3	0	45	1
Gonads†—Ovary	6	2	1	0	14	9	3	1	1	0	25	12
Testis.....	5	3	1	1	0	1	1	0	...	...	7	5
Kidney.....	2	0	2	0	3	2	2	0	2	0	11	2
Oviduct.....	1	0	...	...	2	1	...	...	1	1	4	2
Pericardium.....	2	0	1	0	...	...	2	0	1	0	6	0
Suprarenals.....	1	1	1	0	0	4	...	...	...	...	2	5
Pancreas.....	2	0	...	...	1	1	...	...	...	...	3	1
Heart.....	...	...	...	...	2	0	0	1	1	0	3	1
Gizzard.....	...	...	1	0	2	0	...	...	...	...	3	0
Cloaca.....	...	...	...	...	...	...	1	0	1	0	2	0
Indefinite record.	7	...	...	...	4	1	...	...	2	...	14	...
Totals.....	227	34	109	16	1,207	232	159	35	106	20	1,799	337

\* Group I composed of common pigeons and a few individuals of wild species of genus *Columba*.

Group II composed of pure species, mostly tropical genera (*Turtur*, *Spilopelia*, *Stigmatopelia*, *Oeyphaps*, *Phaps*, *Geopelia*).

Group III composed of *Streptopelia risoria*, *St. alba*, *St. douraea*, and their hybrids.

Group IV composed of generic hybrids of *Turtur orientalis* and *Streptopelia alba* and *risoria*.

Group V composed of generic hybrids of *Stigmatopelia*, *Zenaida*, *Zenaidura*, *Spilopelia* and *Streptopelia*.

† Five cases of "lung suspected" but right or left not designated.

‡ The ovary really ranks sixth (i. e., before "intestines" and "abdominal wall") because the numbers given are based on one sex only. The oviduct takes the place indicated for the same reason.

done with these birds, they were given the best of care during nearly all of this period. This care included: fairly ample, essentially clean and light quarters; abundant and presumably suitable food; the opportunity to mate at maturity (though many were not permitted to rear young); freedom from vermin or body lice in most cases; and protection from extreme cold in heated buildings during the winter.



The data offer some evidence on the question whether the organs of distinct hybrids—crosses involving different genera—are differently susceptible to this particular infection in comparison with the organs of the parent species. There is a high degree of similarity of all these groups. The spleen, liver and lungs take this order in the four groups. The evidence obtained indicates that in pigeons the chief sites of tuberculous infection, or at any rate the infections uniformly considered by us to be tuberculous, are not changed by the fact that these organs are the product of an extremely wide hybridization.

#### SUMMARY

Statistical data are given for the relative extent to which the various organs of 940 Columbidae were infected with tuberculosis or with a macroscopically similar infection. Bacteriologic examinations of these infections were not made, but there can be little doubt that most of these were cases of infection by avian tuberculosis bacilli.

Four of the five groups examined show the spleen, liver and lungs infected in this relative order; the spleen and liver alone include about two thirds of the total number of the obviously infected organs of the body.

The common pigeons present a similar yet appreciably different ranking of infected organs since the relative order for this group probably is: liver, spleen, joints and lungs.

The organs most often infected are apparently also the most intensively or extensively infected organs.

It is suggested that since the order of infection of organs in common pigeons is essentially the same as the order in which the organs of these birds remove intravenously injected bacteria, as shown by Kyes, there is some sort of causal connection between the two facts.

Results recently obtained by other investigators on the distribution of injected manganese dioxide in the fowl also seem to invite the suggestion that the organs which probably remove most of such finely divided nonliving particles and most infecting bacteria from the blood stream are the organs most often infected by tuberculosis.

The ovary is probably more often infected than is the testis in most groups of pigeons; in domestic or common pigeons the data indicate an equal susceptibility of ovary and testis to this infection.

The organs of hybrid birds derived from different genera are probably not changed in their relative susceptibility to tuberculous infection by the mere fact that these organs are of hybrid origin.

## OPSONIC REACTIVATION OF ANTIMENINGOCOCCUS SERUM

LUDVIG HEKTOEN AND RUTH TUNNICLIFF

*From the John McCormick Institute for Infectious Diseases, Chicago*

As the result of a long series of careful tests of the opsonic power of antimeningococcus serum, Alice C. Evans<sup>1</sup> has this to say about reactivation of such serum:

In a recent publication, Kolmer, Toyama, and Matzumami (1918) reported that the phagocytic activity of antimeningococcus serum diminished considerably after heating, or after the addition of 0.2% tricresol, followed by standing at room temperature for 4 days or longer. These investigators reported that the addition of fresh normal human or guinea-pig serum to various antimeningococcus serums as prepared and marketed for administration was found definitely and uniformly to increase their opsonic activity for various strains of meningococci.

According to these investigators, therefore, the labile opsonins are important antibodies in fresh antimeningococcus serum, and they can be restored by the addition of complement. The studies here reported do not confirm those conclusions. Guinea-pig complement has been added to commercial serums in many tests. In some of the tests it was added in a constant ratio of 1:300 in each of the serum dilutions, and in other tests following Kolmer's technic, 1 part of the complement was added to 9 parts of immune serum before the dilutions were made. The results did not show phagocytic action in higher titer of immune serum with complement added than in the controls without complement. Repeated tests have failed to prove that a reaction is obtained in higher titer of commercial serum when complement has been added. These results are in agreement with those of Clough (1919) who recently reported that if antipneumococcus serum had become inactive or feeble as a result of overheating, long preservation, or dilution, the phagocytic activity for pneumococci could not be restored or increased by the addition of complement. An attempt was made to demonstrate opsonins in immune serum by approaching the problem from another angle. Fresh antimeningococcus rabbit serums were heated at 56 C. for 30 minutes and the phagocytic antibodies in the heated and unheated serums were determined in a number of preliminary tests, which indicated that the phagocytic activity of the serums was not diminished by heating.

There is no evidence that the activity of the serum was to any noteworthy degree diminished by the destruction of complement. Contrary to the conclusions of the mentioned authors, the results obtained when complement was added to commercial serum, and when the phagocytic activity of heated and unheated fresh immune serums was compared, indicate that the labile opsonins of antimeningococcus serum play a minor part in promoting phagocytosis as compared with the stronger activity of the tropins. In the words of Zinsser,

Received for publication, July 2, 1921.

<sup>1</sup> Bull. No. 124, Hyg. Lab., Wash., p. 43.

"If thermolabile opsonins as distinct antibodies in immune serum are rendered active by the addition of complement, they are in such low dilution, as compared with the thermostable tropins, that their effect is not measurable.

In the remainder of this paper the phagocytic bodies of antimeningococcus serum will be referred to as tropins."

We would point out here that Zinsser, who is quoted by Evans, by no means favors the view that there is a fundamental difference between labile opsonins and the so-called tropins. His idea is that:

Even in the case of the immune opsonins or bacteriotropins we may think of the participation of two substances—a sensitizer-like one and one comparable to alexin or complement. We may, at least, infer that the full opsonic action of both normal and immune sera is dependent upon the cooperation of two such bodies. It is likely, therefore, that the mechanism of normal and of immune opsonic action may, after all, differ only in quantitative relations between the two.<sup>2</sup>

#### INFECTION AND RESISTANCE

As Evans' results are contrary to what we had been led to believe to be the case from our work with other opsonins, normal as well as immune, bacterial as well as erythrocytic, further experiments have been made which indicate that by the method used the opsonic powers of antimeningococcus serum may be increased markedly (opsonic reactivation) by the addition of small quantities of fresh normal serum. Briefly, our method consists in mixing in small pipets equal quantities of dilutions of antimeningococcus serum, suspensions of washed leukocytes (human), meningococcus suspensions, and of dilutions of fresh normal serum, incubating the mixture at 35 C. for 25-50 minutes when smears are made and the percentage of polymorphonuclear leukocytes engaged in phagocytosis as well as the number of cocci in each leukocyte determined by the study of at least 100 unselected polymorphonuclear leukocytes. Further details about our tests follow.

*Meningococcus Suspension.*—Twenty-four hour growths on goat blood agar are suspended in plain broth just before use and in such quantity as to make the suspension slightly turbid only, the cocci in smears being well distributed over the field.

*Leukocytes.*—Human leukocytes are used, the blood being collected in 2% sodium citrate solution, centrifuged, washed in 0.9% salt solution and suspended finally in about one-half the volume of the washed corpuscles in salt solution. The suspension is used within 2 hours after its preparation.

*Opsonic Mixtures.*—These consist of equal quantities of meningococcus suspension, leukocytic suspension, dilutions of antimeningococcus serum and dilutions of fresh normal human serum. In the controls proper amounts of broth or 0.9% salt solution take the place of one or both serum dilutions. The suspensions and serum dilutions are mixed in pipets and incubated at 37 C. for 25-50 minutes when smears are made and stained with carbol-thionin.

<sup>2</sup> *Infection and Resistance*, 1918, p. 323.

# OPSONIC REACTIVATION OF ANTI-MENINGOCOCCUS SERUM 555

The salt solution suspensions and serum dilutions are kept at room temperature during the preparation of the materials.

No advantage has been noted by incubating the meningococci with serum before adding leukocytes. After 15 minutes' incubation the results appeared to be relatively the same as those after incubation for 50 minutes, the longer incubation giving somewhat higher figures, however.

The number of cocci taken up and the number of cells active in phagocytosis are counted, the count being limited to polymorphonuclears.

Tables 1 and 2 illustrate the results, table 2 showing that normal serum in a dilution of 1:40 may promote in marked degree the opsonic action of antimeningococcus horse serum in a dilution of 1:400.

TABLE 1  
OPSONIC ACTIVATION OF ANTIMENINGOCOCCUS HORSE SERUM WITH NORMAL HUMAN SERUM \*

Opsonic Mixtures Equal Parts of Dilutions of 1:20 of Antimeningococcus Serum and Normal Human Serum, Meningococcus and Leukocyte Suspensions. All Mixtures of the Same Quantity	Meningococcus Strains					
	R		S		T	
	No. of Cocci per Leuko- cytes	% of Leuko- cytes Engaged in Phago- cytosis	No. of Cocci per Leuko- cytes	% of Leuko- cytes Engaged in Phago- cytosis	No. of Cocci per Leuko- cytes	% of Leuko- cytes Engaged in Phago- cytosis
Antimeningococcus serum 1.....	0.4	8	0.4	4	0.6	12
Antimeningococcus serum 1 with normal serum.....	1.4	32	1.0	40	1.4	40
Antimeningococcus serum 2.....	0.2	12	0.0	0	0.3	12
Antimeningococcus serum 2 with normal serum.....	2.1	44	3.0	52	1.9	32
Antimeningococcus serum 3.....	2.4	24	0.64	8	0.1	12
Antimeningococcus serum 3 with normal serum.....	5.2	48	2.3	36	2.6	48
Antimeningococcus serum 4.....	1.3	20	1.3	16	0.1	4
Antimeningococcus serum 4 with normal serum.....	3.6	52	2.0	44	0.75	36
Normal serum only and salt solu- tion.....	0.2	8	0.16	8	0.2	12

\* In each case the dilution of antiserum and of normal serum is 1:80.

Precisely analogous results are obtained in similar tests with normal human serum heated to 56 C. for 30 minutes, on addition of small quantities of unheated human serum (table 3).

## DISCUSSION AND SUMMARY

Our results are definite and impressive. They are in complete harmony with earlier observations<sup>3</sup> showing that opsonic serum, "normal as well as immune owe their full action to a thermostabile

<sup>3</sup> Zinsser reviews the literature well. See also Hektoen, Jour. Infect. Dis., 1909, 6, p. 66. Weaver and Tunncliff, *ibid.*, 1911, 9, p. 130. Bürgers and Meiner, Ztsch. Immunitätsf., 1911, 11, p. 578. Tunncliff, Jour. Am. Med. Assn., 1920, 75, p. 1339.

opsonic substance and a thermolabile complement-like body which greatly promotes the action of the first substance," and confirm the conclusion of Kolmer, Toyama and Matzunami<sup>4</sup> that the opsonic power of antimeningococcus serum may be increased by fresh normal serum. Evidently the method used by Evans is not suited to bring out

TABLE 2  
ACTIVATION OF ANTIMENINGOCOCCUS SERUM WITH NORMAL HUMAN SERUM UNDER  
VARYING QUANTITATIVE RELATIONS

Mixtures			Percentage of Leukocytes Engaged in Phagocytosis
Normal Serum 1:40 + Antimeningococcus Serum 1:40.....			44
Normal Serum 1:80 + Antimeningococcus Serum 1:40.....			40
Normal Serum 1:160 + Antimeningococcus Serum 1:40.....			48
Normal Serum 1:400 + Antimeningococcus Serum 1:40.....			44
Normal Serum 1:800 + Antimeningococcus Serum 1:40.....			16
Normal Serum 1:40 + Antimeningococcus Serum 1:40.....			44
Normal Serum 1:40 + Antimeningococcus Serum 1:80.....			44
Normal Serum 1:40 + Antimeningococcus Serum 1:160.....			40
Normal Serum 1:40 + Antimeningococcus Serum 1:400.....			24
Normal Serum 1:40 + Antimeningococcus Serum 1:800.....			8
Normal Serum 1:40 + Broth.....			12
Broth + Antimeningococcus Serum 1:40.....			12

the activating influence of normal serum on antimeningococcus serum. Here it may be pointed out that Meyer,<sup>5</sup> contrary to the claim of Clough,<sup>6</sup> obtained cleancut activation of the thermostable opsonic element in antipneumococcus serum by normal serum in quantities that by themselves have very little opsonic effect. In short, the view that there is a fundamental distinction between the "labile opsonins"

TABLE 3  
ACTIVATION OF MENINGOCOCCUS OPSONIN IN HEATED NORMAL HUMAN SERUM BY SMALL  
QUANTITIES OF UNHEATED HUMAN SERUM\*

	Percentage of Leukocytes Engaged in Phagocytosis
Mixtures with unheated serum 1 to 4.....	47
Mixtures with heated serum 1 to 4.....	13
Mixtures with unheated serum 1 to 50.....	14
Mixture with heated serum 1 to 4 and unheated serum 1 to 50....	44

\* Other dilutions of unheated serum (1:25, 1:100) gave similar results. The same results were obtained when Locke's solution was used instead of 0.9 % salt solution.

of normal serum and the hypothetic tropins lacks the support necessary to justify its retention, and the use of the word tropin to designate a special opsonic substance, the existence of which has not been demonstrated, would better be dropped.

<sup>4</sup> Jour. Immunol., 1918, 3, p. 156.

<sup>5</sup> Jour. Infect. Dis., 1920, 27, p. 82.

<sup>6</sup> Bull. Johns Hopkins Hosp., 1919, 30, p. 167.

## BLANCHING OF THE SKIN BY SERUM INJECTION IN SCARLET FEVER

F. W. M U L S O W

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In the treatment of scarlet fever with serum from convalescents of scarlet fever, Schultz and Charlton<sup>1</sup> observed, the day following the injection of the serum, that the rash had faded around the point of injection. This blanching appeared to extend farthest along the lymphatics. They also found, by injecting intracutaneously from 0.5 to 1 c c of serum from normal persons or convalescents of scarlet fever, that there was a blanching of the rash for a distance of 1 cm. around the point of injection. This blanching was somewhat irregular in outline, appeared from 6 to 8 hours after the injection and persisted until the general rash had faded. The serums from several other animals did not produce such blanching.

Neumann<sup>2</sup> obtained similar results, and in addition, he found that rashes from other causes, which resemble that in scarlet fever, were not extinguished by convalescent or normal serum. He also found that the serums from cases of diphtheria, measles and other exanthems reacted in the same way as normal serum, but that the serum from scarlet fever patients in the first four days of the disease did not produce the blanching. From these observations Neumann concludes that the blanching phenomenon may be a valuable aid in the differential diagnosis of scarlet fever.

Schultz<sup>3</sup> more recently has confirmed the work of Neumann and his own earlier findings, and states that blanching occurred in 100% of the cases when the injections were made on the second day of the rash, in 78% of 14 cases injected the third day of the rash, in 60% of 5 cases each, injected on the first and fourth day of the rash, and in no case when injected on the fifth day. It is interesting to note that the results are as variable when injections are made on the first day as on the fourth day.

Tron,<sup>4</sup> making similar injections with human serums from various sources in 50 scarlet fever patients, concludes that the blanching phe-

Received for publication July 8, 1921.

<sup>1</sup> Ztschr. f. Kinderheilkunde, 1918, 17, p. 328.

<sup>2</sup> Deutsch. med. Wchnschr., 1920, 46, p. 566.

<sup>3</sup> Acta. med. Scand., 1921, 54, p. 49.

<sup>4</sup> Riforma med., 1921, 57, p. 55.

nomenon has little diagnostic value, since blanching occurred in only 26% of injections made with serums other than those from acute cases of scarlet fever. He confirms, however, the results of the previous investigators in that the serum from scarlet fever patients during the first few days of the disease does not produce the blanching.

In my work blood was drawn from convalescent scarlet fever patients from 21 to 28 days after the appearance of the rash and from the acutely ill during the first 4 days of the rash. The serum was drawn off after the blood had been at icebox temperature for from 10 to 18 hours. The Wassermann and sterility tests were then made and the serum, placed in 1 c c vials, heated at 56 degrees for an hour, and stored in the icebox for as long as 2 months in some instances. It was soon found that the unheated serum reacted in the same way as the heated serum, hence many of the more recent tests have been made with unheated serum. Schultz and Tron also observed that heating of the serum did not influence its action. From 0.5 to 1 c c of the serum was injected intracutaneously into scarlet fever patients during the first 4 days of the eruption. Injections were made in the lower chest region, abdominal wall or thigh. Samples of serum from an acute case of scarlet fever and from a normal person or scarlet fever convalescent were injected at the same time and in the same general region. In some instances as many as 5 injections were made in a patient at one time.

The blanching of the rash in scarlet fever that follows the intracutaneous injection of serum from normal persons or convalescents from scarlet fever, is usually quite definite and involves on an average a circular area about 2.5 cm. in diameter. In some instances, however, the area is as large across as 5 cm. with a very irregular outline, while in other cases it is only 1 cm. across and quite regular. When the blanching is definite it persists until the general rash fades, but in some cases it is only slightly perceptible and soon disappears entirely. The outline of the blanched area is rather irregular but quite definite although frequently the change from the faded area to the surrounding rash is gradual and the border indefinite.

The intracutaneous injection of convalescent serum into scarlet fever patients during the first 4 days of the rash produced definite blanching around the point of injection in 53% of the cases and slight or questionable blanching in 10%. The injection of normal serum produced definite blanching in 40% and slight blanching in 15% of the cases. The injection of serum drawn from scarlet fever patients during



the first four days of the eruption did not produce blanching, except in one instance in which there was a slight or questionable fading of the rash around the point of injection. Often when definite blanching occurred there was a slight redness of the skin where the wheal-like lesions was made during the injection. Storing the serums in the icebox for as long as 2 months apparently did not affect their activity. There was no blanching of the rash in a case of rubella or in the scarlatinoid eruption following the administration of antitoxin when serum from a normal man and from 2 convalescents from scarlet fever was injected. The serum from the case of rubella produced slight blanching in 1 of 3

TABLE 1  
RESULTS OF INJECTIONS WITH SERUMS

Source of Serum	Number of Patients with Scarlet Fever Injected with Each	Number Showing Blanching	Percentage Showing Blanching
Normal Persons:			
1.....	3	2 and 1 ?	40
2.....	3	1	
3.....	2	0	
4.....	3	1 and 1 ?	
5.....	3	1 and 1 ?	
6.....	4	2	
7.....	2	1	
Convalescent from Scarlatina:			
1.....	2	1	53
2.....	2	1	
3.....	2	1 ?	
4.....	3	2	
5.....	13	7 and 2 ?	
6.....	6	4	
7.....	2	1	
Acute stage of scarlatina:			
1.....	2	0	0
2.....	3	1 ?	
3.....	2	0	
4.....	5	0	
5.....	2	0	
6.....	3	0	
7.....	8	0	
8.....	1	0	
9.....	2	0	

injections made in scarlet fever rashes; it caused a reaction, however, the same as serums from 2 scarlet fever convalescents except that one of the latter produced a more marked blanching in the one case where blanching occurred. The table shows the injections and the results in cases of scarlet fever.

#### SUMMARY

While definite conclusions cannot be made from so few observations, it appears that the serum from those acutely ill with scarlet fever rarely produces any blanching effect. The serum from normal persons or

scarlet fever convalescents produces quite a definite blanching in approximately 50% of the cases; there was a taint suggestion of blanching in 12% of the whole number and an entire absence of any blanching in the rest. In considering the practical applications of this test it must be recalled that when the rash is rather faint or fading, blanching cannot be observed. In general, if no blanching occurs when serum is injected into a patient in the acute stage of scarlet fever, the results would mean little, but should blanching occur it would appear that the injected serum is not from a scarlet fever patient in the acute stage of the disease. Although the blanching phenomenon is interesting from the scientific standpoint, it does not appear at present to be of signal value in the differential diagnosis of scarlet fever.

## A STUDY OF THE GONOCOCCUS AND GONOCOCCAL INFECTIONS\*

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The purpose of this work was: first, to devise improved methods of diagnosis of gonorrhea, and, second, to determine whether typing of strains of gonococci could be made. The need of improved methods of diagnosis of gonococcal infections is well known. The advantages to be derived from a typing of the gonococcus, aside from a theoretical interest, lie in the possibilities thus offered of a better specific therapy. The methods of diagnosis which have been studied are both cultural and serologic. Attempts were made to develop a medium on which the gonococcus would grow even if present in small numbers and accompanied by contaminating organisms. This necessitated a study of the factors essential to growth, namely, nutrient substances, and certain conditions of the environment. Diagnosis by means of serologic procedures has included work on the alexin fixation reaction. The intracutaneous test was also studied. Typing of strains of the gonococcus was attempted by the usual fixation and agglutination tests, and also by the method of absorption of agglutinins.

*Growth of Pure Cultures of the Gonococcus.*—Cultural work was first concerned with the growing of stock cultures. The stock cultures consisted of 6 strains obtained from the Cutter laboratories and 10 strains which we had isolated, all of which had been obtained from acute cases of anterior urethritis in men. A number of mediums, which have recently been claimed to possess distinct advantages, were compared from the standpoint of growth favoring properties. In every instance the method of preparing the mediums as described by the authors has been faithfully followed. The solid mediums in the series were used as slants. The results are given in table 1.

As is evident, of mediums employed, testicular agar or testicular agar containing blood or hydrocele fluid proved the most satisfactory. In keeping stock cultures, testicular agar alone gave good

\* The study of the gonococcus which is here reported was made possible by a grant from the United States Interdepartmental Social Hygiene Board.

results. For the invigoration of poorly growing strains, chocolate blood testicular agar was particularly advantageous. Alternation from testicular agar to chocolate blood testicular agar and back again for several generations invariably restored the growth of a weak strain. Testicular agar containing yeast possessed no advantages in growth producing properties over testicular agar alone, in spite of the fact that a vitamine factor may have been supplied. Eberson's semisolid yeast agar, which was advocated because of the property of maintaining viability in the case of meningococcus, gave no growth. Starch agar and tryptamine agar were disappointing. No growth was obtained in any fluid medium. The vigor of stock cultures was maintained most satisfactorily by transplanting at 3-day intervals and keeping at 37 C. Cultures left at room temperature or in the icebox were less active than those kept at incubator temperature.

TABLE 1  
GROWTH FAVORING PROPERTIES OF VARIOUS MEDIUMS

Medium	Growth
Beef infusion agar.....	None
Starch agar (Vedder <sup>1</sup> ).....	Poor
Tryptamine agar (Cole and Lloyd <sup>2</sup> ).....	Fair
Beef infusion agar with 10% hydrocele fluid.....	Fair
Beef infusion agar with 10% blood.....	Good
Testicular agar (Hall <sup>3</sup> ) (Clark <sup>4</sup> ).....	Luxuriant
Testicular agar containing 10% hydrocele fluid.....	Luxuriant
Testicular agar containing 10% blood.....	Luxuriant
Testicular agar containing 10% blood coagulated.....	Luxuriant
(Chocolate blood agar)	
Testicular agar containing infusion of yeast.....	Luxuriant
Semisolid yeast agar (Eberson <sup>5</sup> ).....	None
Beef infusion broth.....	None
Beef infusion broth containing 10% hydrocele fluid.....	None
Beef infusion broth containing 10% blood.....	None
Brain medium (von Hibler <sup>6</sup> ).....	Very scanty
Ground testicular medium prepared like brain medium.....	Very scanty

The period of viability of gonococcus was determined with several mediums which were selected because of the results obtained in the study of their growth favoring properties. Testicular agar and chocolate testicular agar were chosen because of the active growth obtained. Yeast testicular agar was used because of the possibility that the vitamins supplied by the yeast would prove a factor favorable to prolonged growth. Ground testicular medium was also tried, as it offered conditions of environment similar to the brain medium on which

<sup>1</sup> Jour. Infect. Dis., 1915, 16, p. 385.

<sup>2</sup> Jour. Path. & Bacteriol., 1917, 21, p. 267.

<sup>3</sup> Jour. Bacteriol., 1916, 1, p. 343.

<sup>4</sup> Ibid., 1920, 5, p. 99.

<sup>5</sup> Abstr. Bacteriol., 1919, 3, p. 10.

<sup>6</sup> Untersuchungen über pathogene Anaeroben, 1908, p. 85.

Bradley<sup>7</sup> reported successful results with the meningococcus. The agar mediums were used in the form of slants, as preliminary experiments on stab cultures of these mediums showed no recoverable growth after only 3 days' incubation. Determinations on each medium were made in quadruplicate, as 4 strains were used for inoculation, but as the results with all 4 were uniform, they are not given separately. All tubes were held at 37 C. during the entire period of the test and subcultures made on the most favorable medium, chocolate testicular agar, every 2 days. The results are given in table 2.

TABLE 2  
RESULT OF TESTS

Medium	Period of Viability at 37 C.
Testicular agar.....	8 days
Chocolate blood testicular agar.....	8 days
Yeast testicular agar.....	8 days
Ground testicular medium.....	8-12 days

The apparently slight advantage of ground testicular medium over the other mediums was offset by the fact that only scanty growth could be obtained on transplants from this medium. None of the mediums gave promising results from the point of view of prolonging the period of viability.

The effect of temperature on viability was determined with cultures on testicular agar. Cultures were grown for 48 hours at 37 C. and were then placed in the icebox at 4 C., at room temperature at about 20 C., and in the incubator at 37 C. Cultures from each series were viable 8 days and no longer. During the period of test, transplants from tubes kept at 37 C. showed somewhat more luxuriant growth than those of the room temperature and icebox series. However, the actual period of viability was no longer in the series kept at 37 C.

The environmental factors that have been considered of most importance in the growth of the gonococcus are moisture, the physical state of the medium and oxygen tension. Moisture in the atmosphere is generally accepted as a requirement for luxuriant growth. In the present investigation cultures were grown continuously in closed jars containing water. The growth in these jars was uniformly good. On the other hand, cultures kept in the open chamber of the incubator, even though a pan of water was always kept in the same chamber,

<sup>7</sup> Jour. Am. Med. Assn., 1918, 70, p. 1816.

never showed as early or as active growth as those in the closed jars. Saturation of the atmosphere with moisture seems therefore to be an essential factor in growth of the gonococcus.

The advisability of using moist or dry medium is a question on which there is disagreement. McCann,<sup>8</sup> Van Saun,<sup>9</sup> and more recently Jenkins<sup>10</sup> recommend a medium of high water content. Cole and Lloyd<sup>2</sup> consider a moist medium essential, but at the same time allow a certain period of maturation of the medium, during which time they claim that there occurs a concentration of adsorbable hormones at the surface. Hall<sup>3</sup> advises the use of a heavy agar free from excessive moisture. Our work has shown that maximum growth is obtained on a medium containing 2.5% agar from which excessive moisture is removed by 24 hours' incubation at 37 C. and 24 to 48 hours at room temperature. The character of the surface of the medium was found to be an important factor. A hard smooth surface produces good growth, while cultures grow poorly on agar which is soft and easily broken. Water of condensation is not favorable to growth.

The value of oxygen tension as an environmental factor is again a disputed question. Wherry and Oliver,<sup>11</sup> Ruediger,<sup>12</sup> Swartz, Shohl, and Davis<sup>13</sup> all find a reduced oxygen tension favorable to growth of the gonococcus. Chapin<sup>14</sup> obtains better results with an atmosphere in which 10% of air is replaced by carbon dioxide than in ordinary atmosphere. Herrold<sup>15</sup> and Hermanies<sup>16</sup> advocate the growing of cultures in closed systems in the presence of cultures of *B. subtilis*. They find this procedure of particular value for the isolation of cultures. In the present work, the value of reduced oxygen tension was tried by running 2 series of parallel cultures—one grown in a moist atmosphere in which 10% of the air was replaced by carbon dioxide, the other in an identical moist atmosphere without carbon dioxide. No differences were observed in the two series with respect to the abundance or rapidity of growth. Stock cultures and newly isolated cultures were grown in closed systems with cultures of *B. subtilis*. No improvement

<sup>8</sup> Lancet, 1896, 1, p. 149.

<sup>9</sup> Dept. of Health, N. Y., 1913, 7, p. 101.

<sup>10</sup> Jour. Path. & Bacteriol., 1921, 24, p. 160.

<sup>11</sup> Jour. Infect. Dis., 1916, 19, p. 288.

<sup>12</sup> Ibid., 1919, 24, p. 376.

<sup>13</sup> Johns Hopkins Hosp. Bull., 1920, 31, p. 449.

<sup>14</sup> Jour. Infect. Dis., 1918, 23, p. 342.

<sup>15</sup> Jour. Am. Med. Assn., 1921, 76, p. 225.

<sup>16</sup> Infect. Dis., 1921, 28, p. 133.

in growth was noted over that obtained in a moist atmosphere. We must conclude therefore that the value of a reduced oxygen tension has been overestimated.

*Isolation of Cultures of Gonococcus.*—Isolation of cultures was attempted from two types of cases—from acute cases of anterior urethritis in men and from chronic cases in women. The procedure varied according to the type of case.

In acute anterior urethritis in the male, after cleansing the meatus and expressing several drops of pus, cultures were made directly on slants of chocolate blood testicular agar. If the discharge was copious, pure cultures were sometimes obtained directly. Even when contaminating organisms were present in fairly large numbers, a sufficient growth of gonococcus was usually obtained to permit a later isolation. The gonococcus was isolated from 50% of 20 untreated cases of acute anterior urethritis in the male. These cultures were kept on chocolate blood testicular agar for the first 8 or 10 generations, after which they could be transferred to testicular agar.

In isolation of cultures from the cervix uteri in chronic cases of gonorrhea in women, measures were required to inhibit the growth of contaminating organisms. The studies of Churchman,<sup>17</sup> Browning and Gilmour,<sup>18</sup> Drennan and Teague,<sup>19</sup> Dreyer, Kriegler and Walker,<sup>20</sup> Krumweide and Pratt,<sup>21</sup> Gay and Morrison<sup>22</sup> have demonstrated the selective inhibitory action of certain dyes of the triphenylmethane series for a number of organisms. Gentian violet inhibits the growth of staphylococcus and other gram-positive organisms in dilutions which have no effect on gram-negative forms. The inhibitory action of brilliant green for *B. coli* is known. Brilliant green, malachite green, methyl violet and solid green are distinctly bactericidal for streptococcus and to a less extent for staphylococcus. On the basis of these facts, it seemed possible that a dye or combination of dyes might be found which would inhibit the growth of the vaginal flora, at the same time permitting the growth of the gonococcus. Accordingly, a number of dyes were selected for a comparative study of their inhibitory action on the gonococcus, staphylococcus, streptococcus and *B. coli*. These were gentian violet, methyl violet, brilliant green, solid green, malachite

<sup>17</sup> Jour. Exper. Med., 1912, 16, p. 221; 17, p. 373.

<sup>18</sup> Jour. Path. & Bacteriol., 1914, 18, p. 144.

<sup>19</sup> Jour. Med. Res., 1916, 35, p. 519.

<sup>20</sup> Jour. Path. & Bacteriol., 1911, 15, p. 133.

<sup>21</sup> Jour. Exper. Med., 1913, 29, p. 20.

<sup>22</sup> Jour. Infect. Dis., 1921, 28, p. 1.

green, basic fuchsin, pyronin and Spiller's purple. The dyes of the acridine group, although distinctly active against streptococcus and staphylococcus, were not included in the series, as members of this series are known to possess a marked inhibitory action for the gonococcus.

The effect of the dyes was first determined on stock cultures of the organisms in question. Inoculation of cultures was made on testicular or chocolate blood testicular agar to which had been added dye in different concentrations. The  $P_H$  of the medium was 7.6. The action of the dye was shown by the amount of growth obtained after 48 hours' incubation. The results are given in table 3.

TABLE 3  
INHIBITORY ACTION OF DYES ON VARIOUS ORGANISMS

Medium	Dye	Dilution	Gono- coccus	Strepto- coccus	Staphylo- coccus	B. coli
Testicular agar	Gentian violet	1:50,000	—	—	—	++
		1:100,000	+	—	+	++
		1:500,000	+	—	+	++
Chocolate blood testicular agar	Gentian violet	1:50,000	+	—	+	++
Testicular agar	Methyl violet	1:100,000	+	—	+	++
		1:500,000	+	+	+	++
Testicular agar	Brilliant green	1:250,000	+	—	++	++
Testicular agar	Solid green	1:100,000	+	—	+	++
		1:500,000	++	+	+	++
Testicular agar	Malachite green	1:100,000	+	—	+	++
		1:500,000	+	—	+	++
Testicular agar	Basic fuchsin	1:100,000	+	—	+	++
Testicular agar	Pyronin	1:500,000	+	++	+	++
		1:100,000	+	++	++	++
Testicular agar	Spiller's purple	1:100,000	+	++	++	++

It is evident that of the dyes tested none fulfil the requirements which would give ideal conditions for the isolation of the gonococcus. In certain cases there is a retardation, if not complete inhibition of streptococcus and staphylococcus, but no dye inhibits B. coli in a concentration which permits growth of the gonococcus. Gentian violet, solid green, and malachite green have no inhibitory action on the gonococcus in a dilution of 1:500,000. Staphylococcus is partially inhibited by solid green and malachite green. As the gram-positive cocci seem to be the most difficult of separation from the gonococcus in primary cultures, mediums were prepared containing separately gentian violet, malachite green, and solid green.



The nutrient base of the mediums used in isolation was testicular agar. To this was added in some cases 10% blood, which was coagulated to make chocolate blood agar, in other cases 10% hydrocele fluid. It was found that the hydrocele agar was the more satisfactory, since the chocolate blood medium was too opaque to permit the fishing of single colonies. For isolation plates were used. Cultures were taken from within the cervix; pus was smeared over the surface of the medium and the plates were incubated immediately. Plates were examined after 2, 3, and 4 day incubation. A known culture of gonococcus was planted on each series of plates to control the growth producing properties of the medium. Good growth was obtained with this control culture on every medium. The results of cultures made on various mediums will be given.

*Chocolate Blood Testicular Agar Containing Gentian Violet 1:500,000.*—Cultures were taken in twenty-nine cases. Smears for microscopic examination made at the time cultures were made showed only one case in which intracellular gram-negative diplococci were demonstrated. Five showed many extracellular gram-negative diplococci. All were considered clinically chronic cases of gonorrhea. In 10 cultures of this series, the 48-hour growth gave colonies which on microscopic examination were gram-negative diplococci, morphologically typical of gonococcus. Fishings made from these 10 plates on chocolate blood testicular agar gave in 3 cases growths which appeared to be cultures of gram-negative diplococci. We were unable to keep these cultures after 3 or 4 generations, however. In many cases, colonies which were typically gram-negative in their staining properties when fished from the plates containing gentian violet gradually developed a gram-positive character with successive transplants on chocolate blood testicular agar containing no gentian violet. As no pure cultures were isolated from this series, we cannot say that the morphologically typical organisms occurring on the primary cultures were gonococci. However, in this and succeeding series, the growth of biscuit shaped gram-negative diplococci from a gonorrheal discharge was considered at least suggestive of gonococcus.

*Chocolate Blood Testicular Agar Containing Gentian Violet 1:500,000 and Soap 1:2,000 (Loco Castile Soap).*—This medium was prepared like the other except that a solution of soap was added in the hope of approximating conditions obtained by Avery<sup>23</sup> with the

<sup>23</sup> Jour. Am. Med. Assn., 1918, 71, p. 2050.

influenza bacillus. Of 16 cultures, none gave positive microscopic evidence of gonococcus. Colonies of gram-negative diplococci were obtained in the primary cultures from 4 cases but no pure cultures were isolated.

*Chocolate Blood Testicular Agar; No Dye or Soap.*—As controls on the two series mentioned in the previous paragraph, cultures were made on chocolate blood testicular agar with no dye or soap, of 6 cases negative according to microscopic examination. Colonies of gram-negative diplococci were obtained in primary culture from 3 cases. No pure cultures were isolated.

*Hydrocele Testicular Agar Containing Gentian Violet 1:500,000.*—Of 30 cultures, 4 were positive according to microscopic examination made at the time of cultivation. In 8 of these cases gram-negative diplococci were obtained on the primary culture. No pure cultures were isolated.

*Hydrocele Testicular Agar Containing Solid Green 1:500,000.*—Cultures were made from 8 cases. None was positive on microscopic examination. One gave gram-negative diplococci in primary culture. No pure cultures were isolated.

*Hydrocele Testicular Agar Containing Solid Green 1:500,000; Suspension of Yeast 1:1000.*—A suspension of yeast was added in the hope of encouraging the growth of the gonococcus by supplying vitamins. The results were no different from those without yeast.

*Hydrocele Testicular Agar Containing Malachite Green 1:500,000.*—Cultures were made from 2 cases. No gram-negative diplococci were obtained.

*Hydrocele Testicular Agar, No Dye or Yeast.*—Parallel plates containing no dye were inoculated from the same cases as those of the before-mentioned series. Gram-negative diplococci appeared in a slightly lower percentage of plates. No pure cultures were obtained. Contaminating organisms were present in somewhat greater numbers and appeared earlier than in plates containing dye.

While the results show that the presence of dyes in the mediums inhibited the growth of contaminating organisms to a slight extent, the inhibition was nevertheless not sufficient to permit the isolation of the gonococcus. However, the conditions of the experiment were difficult, since of 91 specimens taken, only 5 were positive on microscopic examination. With the exception of these 5 cases, it is impossible to say definitely whether the failure to obtain pure cultures was

due to the fact that the organisms were not present in the discharge or whether sufficiently favorable conditions of growth were not provided. However, the fact that pure cultures were not obtained from the 5 cases in which gonococci were demonstrated microscopically would indicate that the conditions of growth were not adequate for the gonococcus. Moreover, the appearance of colonies of typical gram-negative diplococci in primary cultures, subcultures of which were impossible to grow, is also indicative of the need of perfecting the medium before isolation from chronic cases may be accomplished. The results obtained from the foregoing series, namely, the greater percentage of gonococcus-like colonies and the lower percentage of contaminating organisms on plates containing dye, could indicate that the possibility of successful isolation of the gonococcus lies in the perfection of a selective medium.

#### IMMUNITY TESTS IN GONOCOCCAL INFECTIONS

*The Alexin Fixation Test in Gonorrhea.*—Before attempting to determine the value of the alexin fixation test in the diagnosis of gonorrhea, a number of antigens were prepared and tested for their efficiency. All of these antigens were polyvalent, representing in every case growth from eight strains.

The first series of antigens were suspensions of 48-hour growth of gonococcus in normal salt solution. These were allowed to autolyze at different temperatures by shaking at room temperature for from 6 to 8 hours with subsequent standing in the icebox for 48 hours, by allowing to remain in the incubator for 48 hours, and by heating to 56 C. for 2 hours. Five-tenths per cent, phenol was added to all of the suspensions. After autolysis, tests were made on the inhibitory and antigenic properties of each whole preparation and also on the supernatant fluid obtained by centrifugalization of a portion of each with an immune serum. No appreciable differences were observed in any of these antigens. Neither did the supernatant fluid differ from the whole suspensions. In every case, 2 antigenic units equaled from  $\frac{1}{4}$  to  $\frac{1}{5}$  the inhibitory dose.

Other antigens prepared and tested were: a suspension of organisms subjected to N/10 NaOH one-half hour with subsequent neutralization with N/1HCl; organisms extracted with alcohol and ether according to the method recommended by Smith and Wilson<sup>24</sup>; an antigen dialyzed according to the method recommended by Wadsworth and

<sup>24</sup> Jour. Immunol., 1920, 5, p. 499.

Maltaner<sup>25</sup> for use with *B. tuberculosis*. Of these 3, the first 2 were relatively nonantigenic, while the third was slightly more inhibitory to alexin than the antigens of the first series. Therefore, in all fixation tests used for the purpose of diagnosis, the antigens used was a polyvalent suspension of organisms in normal salt solution, phenolated and autolyzed at 56 C.

Fixation tests of serum of gonorrheal patients were made using the antsheep hemolytic system, 2 units of alexin, fixation at 37 C. for one hour, with further incubation of one-half hour after the addition of sensitized cells. Eighty serums were tested. These are classified and the results given in table 4. A ++ reaction represents complete inhibition.

TABLE 4  
RESULTS OF FIXATION TESTS

Type of Serum	Total Number	Positive	Negative
Gonorrhea:			
Early acute.....	4	2 + -	2
Cured acute 8 weeks after onset.....	2	2 + +	0
Chronic.....	9	4 + + 2 +	3
Arthritis.....	1	1 + +	0
Posterior urethritis.....	1	1 + +	0
Doubtful diagnosis.....	2	0	2
Tuberculosis.....	16	1 + +	15
Syphilis.....	16	0	16
Respiratory, skin and other diseases.....	20	1 + -	19
Normal.....	9	0	9

It is difficult to draw conclusions on the efficiency of a test when so small a series of cases is represented. Nevertheless, certain facts are evident. Early acute cases gave negative or slightly positive reactions. Chronic cases, including gonorrheal arthritis, were positive in 72.8%. Serums taken immediately after cure was established clinically were positive. Normal serum and serum from diseases other than gonorrhea were negative, with the exception of a ++ reaction in one tuberculosis patient. No serum from cases of meningitis was available. The cross reaction so commonly observed between gonococcus and meningococcus could therefore not be tested with meningitis serum. A number of ++ gonorrheal serums, however, were tested against a meningococcus antigen and were found to give negative or at the most slightly positive reactions.

These results corroborate those of other investigators, among whom may be mentioned Teague and Torrey,<sup>26</sup> Watabiki,<sup>27</sup> Kolmer and

<sup>25</sup> Jour. Exper. Med., 1921, 33, p. 119.

<sup>26</sup> Jour. Med. Res., 1907, 17, p. 223.

<sup>27</sup> Jour. Infect. Dis., 1910, 7, p. 159.

Brown,<sup>28</sup> Irons and Nicoll,<sup>29</sup> and Smith and Wilson.<sup>24</sup> The fixation test must be considered a valuable aid in the diagnosis of all except the early acute cases of gonorrhea.

Agglutination and precipitin tests were not tried in diagnosis, since these tests gave less distinctly positive results with known antigonococcus immune serum than did the fixation reaction.

*The Intracutaneous Reaction in Gonorrhea.*—Bruck,<sup>30</sup> Köhler,<sup>31</sup> and Irons<sup>32</sup> studied the reaction of gonorrheal patients to the cutaneous and intracutaneous application of preparations of gonococcus. While their studies indicate that these tests are of diagnostic value, the results were not found entirely reliable, especially in certain types of gonorrhea. Moreover, according to the work of Irons, positive reactions often occur in gonorrheal patients on the application of a preparation of meningococcus. A cross reaction was also obtained with gonococci in meningitis patients. It seemed possible that an improvement might be made in this test by using a different preparation of antigen. The results of Gay and Force<sup>33</sup> with typhoidin and of Gay and Minaker<sup>34</sup> with a preparation of meningococcus suggested the use of a similar antigen in gonorrhea. It was hoped that this antigen would prove more active and more specific than the preparations used by Bruck, Köhler and Irons.

The technic of preparation of this antigen was as follows: Seventy-two hour cultures of 8 strains of gonococcus on testicular agar were washed off in water. The aqueous suspension was placed in the incubator for 48 hours to allow autolysis of the organisms. From 10 to 15 volumes of 95% alcohol were then added. The mixture was shaken and allowed to stand for 24 hours. The supernatant fluid was removed and the precipitate washed once in 95% alcohol and twice in absolute alcohol. The precipitate was then shaken with ether, the ethereal suspension poured on hard filter paper and washed with absolute ether. The residue was dried over sulphuric acid at 40 C. When apparently dry, the product was ground to a powder in an agate mortar and kept in a desiccator at 40 C. until of constant weight. For use this powder was suspended in phenolated salt solution. The

<sup>28</sup> Ibid., 1914, 15, p. 6.

<sup>29</sup> Ibid., 1915, 16, p. 303.

<sup>30</sup> Deutsch. med. Wchnschr., 1909, 35, p. 470.

<sup>31</sup> Wien. klin. Wchnschr., 1911, 24, p. 1564.

<sup>32</sup> Jour. Infect. Dis., 1912, 11, p. 77.

<sup>33</sup> Arch. Int. Med., 1914, 13, p. 471.

<sup>34</sup> Jour. Am. Med. Assn., 1918, 70, p. 215.

optimum concentration was found to be such that 0.05 c c contained 0.0066 mg. of dried powder. As a control a similar preparation of meningococcus was made.

Intracutaneous injections of 0.05 c c of this suspension were given to a series of patients with gonorrhea and a series of normal individuals. A white wheal was the immediate result of injection. The reaction was considered positive if 48 hours after injection there was a marked area of erythema and distinct induration. A slight erythema was probably due to the toxicity of the preparation and could not be considered as a positive reaction. The results are given in table 5.

TABLE 5  
RESULTS OF INJECTIONS WITH GONOCOCCIN AND MENINGOCOCCIN

Type of Case	Number	After Injection with Gonococcin			After Injection with Meningococcin		
		Erythema Induration	Slight Erythema	No Reaction	Erythema Induration	Slight Erythema	No Reaction
Acute epididymitis.....	1	0	0	1	0	0	1
Chronic double epididymitis.....	1	1	0	0	1	0	0
Prostatitis.....	3	1	2	0	2	1	0
Arthritis.....	1	0	1	0	0	1	0
Acute anterior urethritis 1 week cured.....	1	1	0	0	1	0	0
Anterior urethritis 3 mos. and 2 years.....	4	0	1	3	0	1	3
Normals.....	18	1	1	16	2	1	15

From the small series of cases tested, gonococcin cannot be considered to constitute an aid in diagnosis. Three cases of chronic gonorrhea gave positive reactions with gonococcin, but three equally strong positive reactions were obtained with meningococcin. In general, the reactions with meningococcin were identical with those with gonococcin both in gonorrheal cases and in normal cases. In view of these facts, no further work was done with the intracutaneous reaction.

*Typing of Strains of Gonococci.*—Sixteen strains of gonococcus were used in the work on typing. A strain of meningococcus was also included in this study as a control. The strains of gonococcus exhibited no cultural differences. Their fermentative reactions were also identical—all strains giving slight acidity on a dextrose agar with Andrade's indicator. The procedures by means of which typing was attempted were the alexin fixation and agglutination reactions and also absorption of agglutinins of immune serums.

Immune serums were obtained by injection into rabbits intraperitoneally with washed cultures of individual strains. Washed cultures were found to be much less toxic than suspensions of growth which had not been washed. The dosage was gradually increased from one-half culture to 8 to 10 cultures on testicular agar slants. Fifteen to 20 injections were given at intervals of from 3 to 5 days. The production of an immune serum was difficult as the injections were in many cases followed by loss of weight and death of the animals.

Fixation reactions were made with all serums against antigens prepared from each individual strain. Antigens were prepared according to the procedure adopted in the fixation tests reported in the work on diagnosis, except that, of course, in these cases each antigen comprised only one strain. A serum was used for classification only when its fixation titer with the specific strain was at least 1:400.

The results of fixation of each serum with its specific strain and with the heterologous strains showed no characteristic or consistent differences in titers with different antigens. Of 8 serums produced by immunizing against individual strains, 3 gave slightly stronger reactions with the specific than with the heterologous antigens, while 5 gave stronger reactions with heterologous antigens. All serums gave positive reactions with all the gonococcus antigens, and whatever differences there were between the individual strains were not sufficiently marked or consistent to justify a classification on this basis. Any differences in titer of the individual antigens seemed to be due to the efficiency or lack of efficiency of the individual antigens rather than to any specific relationship between serum and antigens. Certain antigens gave a uniformly high titer with all serums, while certain others were uniformly low. A comparison with a meningococcus antigen showed that the titer was in no case so high as with the gonococcus antigens, though the reaction was positive in low dilutions. Normal serums gave negative reactions with all antigens.

The agglutination reaction was also used as a basis of an attempted typing of strains.

Serums immune to 8 individual strains were tested against the eight antigens. Antigen was prepared by washing off a 48-hour culture in salt solution containing 0.4% formalin, centrifugalizing the suspension and resuspending the sediment in formalinized salt solution. The tendency of cultures to autolyze required that the growth to be used for an agglutinating antigen should be taken from fairly dry medium

with no water of condensation. The period of agglutination was 2 hours at 56 C. and overnight in the icebox. The average agglutination titer of the serums was from 1:600 to 1:1200.

The results of these tests gave no further basis of typing than did the fixation reactions. No groups were obtained. With these tests, also, the specific strain often gave a less marked reaction than the heterologous strains. With several serums a slightly positive reaction was obtained with meningococcus. The reactions were in no case so marked as with the gonococcus, however.

Whether the lack of typing obtained by the fixation and agglutination reactions was due to the inadequacy of these tests for this purpose or to a real similarity of the strains was not demonstrated. Further work on the question of relationship between the individual strains was therefore undertaken, using the method of absorption of agglutinins—a procedure by means of which Torrey<sup>35</sup> established his 3 groups and according to which Hermanies<sup>16</sup> has recently defined 6 groups.

The technic was essentially that given by Hermanies. The dilution of serum was determined, from which all agglutinins were absorbed by the specific strain. Having established this, a somewhat higher dilution such as 1:40 instead of 1:20, for instance, was used for absorption by the heterologous strains. The antigens used for absorption were the washed sediment from the growth of four to six testicular agar slants. Antigen for absorption was always used in excess. Serums were absorbed at 56 C. for from 6 to 12 hours and placed in the icebox overnight. On the following morning, the serums were centrifugalized and the clear supernatant fluid used for the test with the immunizing strain of the serums as the antigen. Table 6 indicates the results of absorption tests on seven serums.

It appears from the foregoing experiments that there is no evidence of grouping among the 16 strains of gonococci used. It would seem from the absorption of serum G 1, that strains G 1, G 3, G 4, G 5, G 6, G 11, G 12, G 14, G 15, and G 16 are of one group, as they all remove the major agglutinin from the serum immunized to G 1. It would then be expected that if a serum immune to any one of these strains should be absorbed with the 16 strains that identical results would be obtained. Rabbits were therefore immunized to strains G 11 and G 16. From the absorption of serum G 11, we find that strains G 7, G 9, G 10, and G 11 remove the major agglutinins and from the

<sup>35</sup> Jour. Med. Res., 1907, 16, p. 329.



- absorption of serum G 16 that strains G 3, G 9, G 10, G 14, and G 16 remove the major agglutinins. It can be seen that the results are not identical: many of the strains which removed the agglutinins from serum G 1 have failed to remove the agglutinins from serums G 11 and G 16; strains also removed the agglutinins from the latter serums which failed to remove the agglutinins from the former serum.

Two rabbits were immunized against strain G 8. The absorption experiments with these serums gave dissimilar results, as shown in table 6. Other serums were absorbed, but the agglutinins were removed with no uniformity.

The present work has added nothing to the question of typing of the gonococcus. Our results with fixation and agglutination tests

TABLE 6  
RESULTS OF ABSORPTION EXPERIMENTS

Absorbing Strains	Immune Serums						
	G 1 Serum	G 8 Serum 1	G 8 Serum 2	G 9 Serum	G 11 Serum	G 13 Serum	G 16 Serum
G 1	+	—	—	—	—	—	—
G 2	—	—	—	—	—	+	—
G 3	+	+	—	—	—	+	—
G 4	+	—	—	—	—	—	—
G 5	+	—	+	—	—	—	—
G 6	+	—	—	—	—	—	—
G 7	—	+	+	—	+	+	—
G 8	—	+	+	—	—	—	—
G 9	—	+	—	+	+	—	+
G 10	—	+	+	+	+	+	+
G 11	+	—	—	—	+	—	—
G 12	+	—	—	—	—	+	—
G 13	—	—	—	—	—	+	—
G 14	+	—	+	—	—	—	+
G 15	+	—	—	—	—	—	—
G 16	+	—	—	—	—	—	+

+ indicates that the agglutinins for the immunizing strain of the serum are removed.

on unabsorbed immune serums agree with those of Kolmer and Brown,<sup>28</sup> Wollstein,<sup>36</sup> and Thomsen and Vollmond,<sup>37</sup> none of whom obtained an absolutely clear-cut differentiation between gonococcus and meningococcus, nor a satisfactory grouping of gonococcus strains. On the other hand, Torrey,<sup>35</sup> Watabiki,<sup>27</sup> Pearce,<sup>38</sup> and Jøtten<sup>39</sup> found differences between strains by means of agglutination and fixation reactions. In regard to the tests on absorbed serums, our results differ from those of Torrey<sup>35</sup> and Hermanies.<sup>16</sup> We have not been able to define any groups among our 16 strains of gonococci, absorption of agglutinins having taken place without uniformity.

<sup>28</sup> Jour. Exper. Med., 1907, 9, p. 588.

<sup>37</sup> Compt. rend. Soc. de Biol., 1921, 84, p. 326.

<sup>38</sup> Jour. Exper. Med., 1915, 21, p. 289.

<sup>39</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1921, 92, p. 9.

## CONCLUSIONS

Gonococcus stock cultures were found to grow satisfactorily for all routine work on testicular agar. Chocolate blood testicular agar was found to be a useful medium for increasing the vitality of a weakly growing culture. Environmental requirements of the organism included moisture of the atmosphere but not a reduced oxygen tension.

Isolation of cultures from acute cases of anterior urethritis in men was most successfully accomplished on chocolate blood testicular agar. No pure cultures of gonococci were isolated from chronic cases of gonorrheal endocervicitis, although single colonies of organisms morphologically typical gonococci were obtained on plates of hydrocele testicular agar containing certain members of the triphenylmethane series of dyes as an inhibitor of contaminating organisms.

The alexin fixation test serves as an aid in diagnosis, but it should be still considered rather as confirmatory evidence than as an independent basis of diagnosis. It is of little value in early cases, as might be expected. A nonspecific reaction was obtained on the intracutaneous injection of a preparation of gonococci. A like reaction was obtained in gonorrheal patients on the injection of a preparation of meningococci.

No typing of strains of gonococcus was obtained by means of the alexin fixation and agglutination reactions or by means of the method of absorption of agglutinins.

## DIPHTHERIA CARRIERS AMONG MASSACHUSETTS SCHOOL CHILDREN

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A study of nose and throat cultures from over 8,000 school children of Massachusetts, from 5 to 15 years of age, has been made during the past year and a half. The cultures were taken in 35 cities and towns throughout the state, as a rule, under the direction of the school physicians. Nose and throat cultures were taken from each child. They were taken because a noticeable number of cases of diphtheria had occurred in the schools. They would, therefore, be considered contact cultures although probably many of the pupils did not come in contact with the cases. In some instances cultures were taken from all the pupils in a school, in others, from the pupils in a single room, when the cases were limited to one room.

The 8,389 cultures received were grown on Loeffler's blood serum having a  $P_H$  value of 7.2. After from 14 to 18 hours' incubation at 34° C. they were examined for diphtheria bacilli, the smears being stained with Loeffler's methylene blue. All of the cultures were examined by the same bacteriologists. Of the 8,389 cultures examined, 41 positives were obtained, or approximately 0.49%. The highest percentage of positives in any one group was 13, when 4 positives were found in 31 cultures from one school room.

Virulence tests were made on 38 cultures isolated. Unfortunately, the other 3 cultures were lost before tests could be made. Guinea-pigs weighing from 200 to 300 gm. were inoculated subcutaneously with 1 c.c. of a 48-hour broth culture of the bacillus, the control animal receiving 500 units of diphtheria antitoxin before the broth culture. Two of the 38 cultures tested were found to be nonpathogenic. Approximately 95% were virulent, killing the unprotected guinea-pigs in from 48 to 60 hours.

There was no evidence that cultures diminished in virulence, because some of the cultures had been grown on artificial mediums for 6 months without losing their virulence.

Many diphtheroid bacilli were encountered during the examinations. The familiar Hofmann bacillus gave little trouble but other bacilli more closely resembling diphtheria bacilli were frequently found. In these bacilli polar granules were often observed, but when studied in young cultures they were found to show deviations from the true diphtheria bacillus, especially in the grouping and rate of growth. As it is the custom of this laboratory to examine and report on young (2-8 hour) cultures of diphtheria bacilli in the routine diagnosis of diphtheria, the laboratory workers are very familiar with the appearance of young diphtheria forms. Therefore, differences were readily noted in the case of diphtheroids, and it was not necessary to delay the report of the school cultures more than a few hours. Certain bacilli resembling the B form of Wesbrook's types have been tested in the past for virulence and all have been found avirulent for guinea-pigs. These somewhat suspicious bacilli have been considered by us as diphtheroids, not avirulent diphtheria bacilli. The two cultures tested which did not kill the guinea-pigs may have been avirulent diphtheria bacilli because in morphology and cultural characteristics they appeared typical, but it is quite possible that they were distinct species and that, had we been more discriminating, morphologic differences from the true diphtheria bacillus might have been noticed. Our opinion is that avirulent diphtheria bacilli are rare. The results obtained by us are quite different from those of some other observers, notably Moss, Gelian and Guthrie,<sup>1</sup> who found 85 positive throat cultures among 800 school children of Baltimore, and of the 64 cultures tested 87.5% were avirulent. However, it does not seem safe to assume a similar distribution elsewhere. Examination of over 6,000 contacts made by us in 1915 showed only 0.6% positive. It would appear that among large groups of school children in Massachusetts less than 1% harbor diphtheria bacilli, virulent and avirulent.

We are aware of the fact that a single culture from a throat is not conclusive evidence, but there has been no epidemiologic evidence that we have missed many carriers; in most instances, isolation of the carriers detected has led to a cessation of the cases in the schools. Some of the carriers reported were said to show some symptoms of diphtheria and were actually missed cases rather than healthy carriers. The sphere of influence of one carrier or missed case seems to be

<sup>1</sup> Bull. Johns Hopkins Hosp., 1920, 31, p. 388.

considerable and the detection of the relatively small number of these leads to a diminution of the cases occurring in the schools. The taking of school cultures appears to be of distinct value.

#### SUMMARY

In Massachusetts, school children, among whom cases of diphtheria occurred, showed a small number of carriers of virulent diphtheria bacilli. Of a total of 8,389, only 41 or approximately 0.49% positive cultures were obtained. Of the 38 cultures tested for virulence, approximately 95% were virulent.

Avirulent diphtheria bacilli, said to be common in some places, are uncommon in this state. Only 2 cultures were found which were considered identical with the diphtheria bacillus in morphology.

Diphtheroid bacilli are common, but a careful study of the morphology and cultural characters shows differences from the true diphtheria bacillus and makes many virulence tests unnecessary. Familiarity with young cultures of diphtheria and diphtheroid bacilli is very helpful.

# DYSENTERY-LIKE DISEASES (PARADYSENTERY, PARATYPHOID) IN CHILDREN AND THEIR CAUSES

ONE PLATE

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Bloody or purulent stools mixed with mucus are more commonly observed in children than in adults. The condition may be relieved in a few days or may by degrees increase in intensity until grave symptoms of intoxication ensue and in some cases severe fulminating symptoms develop with death on the second or third day. During the past 9 years I have studied in our clinic several hundred such cases, from 334 of which the 5 kinds of ordinary dysentery bacilli have been isolated, while in 73 organisms have been obtained which belong apparently to a hitherto undescribed species.\* From a few of the cases I obtained cultures of *Bacillus paratyphosus* and a bacillus allied to the paratyphoid group. I have also cultivated Morgan's<sup>1</sup> bacillus, and a variety similar to the strains isolated by Leiner,<sup>†</sup> although I could not confirm their claims that they bear any relation to the clinical condition. This paper is a report on those organisms which I believe are new. Those that resemble ordinary dysentery bacilli I shall speak of as paradysentery bacilli, and those that resemble paratyphoid bacilli as paratyphoid-x bacilli. The reason for these terminologies will be evident later.

## PARADYSENTERY GROUP

In the early stages of the disease the organism may be isolated almost in pure culture, but in most cases it is associated with the colon bacillus. As the disease progresses its isolation becomes more difficult.

Several different strains have been obtained which may be divided into two kinds, according to their behavior toward carbohydrates, having common characteristics otherwise.

The form of the colonies in the agar plate is somewhat larger than that of the true dysentery bacillus. Moreover, besides the regularly formed colonies, such as are seen in cultures of dysentery bacilli, this organism forms also thinner, somewhat larger colonies, a trifle elevated in the center, with quite irregular edges and sometimes with

Received for publication July 16, 1921.

\* Of 7 cases of dysentery in children examined in Chicago in the summer of 1921, 4 yielded the Hiss' bacillus, 1 the Shega bacillus and in 2 the cultures proved negative.

<sup>1</sup> Brit. Med. Jour., 1907, 2, p. 15.

a kind of lobular outline as if undergoing disintegration. When first isolated from the feces, the colonies are usually irregular. This is one of the characteristics by which this group can be differentiated from the true dysentery group.

*Morphology.*—The size is about the same as that of the colon bacillus, and the organisms usually occur singly, although long chains are sometimes seen extending throughout half the field. The organism stains well with anilin dyes, intensely at the ends and less so in the middle. There are no flagella.

*Motility.*—The organisms have no proper motility, although they have molecular movement.

*Biologic Characteristics.*—In a gelatin stab growth is obtained along the stab line, but there is no liquefaction.

Abundant growth is obtained around the stab canal in glucose agar, but there is no gas production.

Good growth is obtained in 1 to 2% Witte peptone solution, and there is no scum formation after continuous prolonged cultivation. There is a more distinct sedimentation of the organisms, than in the case of the dysentery bacilli.

No indol has been demonstrated, even in 1 to 2% Witte peptone solution.

New, freshly isolated strains coagulate milk after from 2 to 3 weeks' cultivation; older cultures after repeated subcultures showed coagulin in 10 to 14 days. Coagulation begins at the bottom and gradually progresses throughout the upper layer. It is complete in 3 to 7 days, when the clear solution is separated out.

Litmus whey is turned red in 24 hours, violet in 3 to 5 days, and red again within the next 2 to 4 days; or it may be violet or red within 24 hours, then blue and finally once more violet or red.

Litmus nutrose glucose solution develops acid and red color, with coagulation after 24 hours.

Carbohydrate decomposition was studied in a solution containing mannite, etc., in a proportion of 1.3% to 1% Witte peptone solution, to which litmus was added as indicator. Mannite and dextrose were split into acid after one day.

Dextrin and maltose were split by both varieties of paradysentery bacilli, but the first type renders these sugars acid the following day, while the second type changes the color after 1-2 weeks; that is, within

several days the color changes into violet and after a longer time turns into red. In general, maltose is somewhat more quickly decomposed than dextrin.

Saccharose and lactose are slowly decomposed by both groups, requiring 2 to 3 weeks; the color first becomes violet, finally red.

Comparison of the carbohydrate reactions only of the paradysentery and the true dysentery bacilli shows the first type of the former group to be apparently indistinguishable from the type 4 (Flexner) and the second from type 2 (Y), but the paradysentery bacillus when kept in culture for many weeks behaves, as has been stated, like the colon bacillus. I have therefore divided the paradysentery strain into 2 types: A, which decomposes maltose and dextrin within 24 hours, and B, which causes no change whatever for several days.

*Differentiation of These Two Varieties of Bacilli from Other Familiar or Similar Organisms.*—Differentiation from similar organisms, such as typhoid, paratyphoid, and other motile bacilli is very simple. Among the colon varieties there is one which decomposes dextrose into acid but forms no gas, and after a few days coagulates milk, but this can be differentiated from our organisms by the time required for the coagulation of milk or the splitting of the carbohydrate, through the forms of colony, and also by means of agglutination reactions. The differentiation from the dysentery bacillus is made by means of agglutination reaction with immune serum, the form of colonies, and by the coagulation of milk. It is true that in the literature there are a number of bacilli isolated from patients with dysentery-like symptoms that resemble ours, for example, those of Duval,<sup>2</sup> Torrey<sup>3</sup> and Leiner,<sup>4</sup> but while the organism described by Duval resembles ours with respect to the behavior on lactose, Duval's bacillus did not coagulate litmus milk and agglutination placed it in the typhus group while my paradysentery bacilli coagulate milk and differ in agglutination from the typhoid group. Torrey's bacillus C also did not coagulate litmus milk, although his older strains sometimes showed coagulation. But to exactly differentiate varieties of similar bacilli requires comparative methods. Leiner's bacillus resembles my strains in its coagulation of milk and carbohydrate reactions, but the colonies are knobbed

<sup>2</sup> Jour. Am. Med. Assn., 1904, 43, p. 381.

<sup>3</sup> Jour. of Exp. Med., 1905, 7, p. 385.

<sup>4</sup> Centralbl. f. Bakteriol., I, O., 1907, 43, p. 783.



and agglutination places it among the saprophytic colon bacilli. It appears, therefore, that the organisms in question represent an independent, specific, pathogenic bacterium.

In 56 of the 73 cases from which the paradysentery bacillus was isolated type A was obtained, in 17 type B. Although type B was so seldom found, it is an interesting strain, as shown by comparison of the carbohydrate reactions of the 2 types with those of the dysentery bacillus, type A resembling type 4 of dysentery bacillus (Flexner), and type B resembling type 2 of dysentery bacillus (Y).

#### IMMUNOLOGIC RELATIONS

*Agglutination.*—The strains which I isolated were agglutinated by the serum of the patients from which they were obtained in dilutions of 300:500 and not infrequently 1,000, but were not agglutinated by the serums of other patients or normal persons. The agglutination titer was in general greater than that usually shown by the dysentery serums for dysentery strains. The 2 types of paradysentery bacillus were affected in practically the same way by the patients' serums, hence they cannot be differentiated from each other by agglutination reactions, although their biologic properties are different. The same holds true of the dysentery bacilli of lower classes, such as 4 or 5 types, the types of which cannot be differentiated by agglutination reactions. Strains of the paradysentery bacillus which have been maintained by cultivation for a long time have the property of auto-agglutination, a phenomenon observed among other pathogenic bacteria.

*Complement fixation.*—The extract of both types of the paradysentery bacillus fixes complement in the presence of homologous serum.

*Virulence for Animals.*—The paradysentery bacillus, whether living or killed, is virulent for all ordinary laboratory animals when injected intraperitoneally or into a vein (1-2 slant agar culture for young rabbits, 3-5 mg. for guinea-pigs, and 0.2-1 mg. for mice), but the virulence is not strong nor definite. Sections of the animal tissue showed no significant changes in the intestinal wall. In this respect the organism is analogous to the so-called acid dysentery bacilli.

#### CLINICAL

Of the 73 children from whom the paradysentery bacillus was isolated, the youngest was 9 months old and the oldest 13 years; I

have also seen two adult cases. The disease was prevalent at all times, but was most frequent in the summer months.

*Symptoms and Course.*—The disease caused by the paradysentery bacillus is difficult to differentiate clinically from that caused by the true dysentery bacillus, except that in the case of the former the intestinal symptoms, as well as the general symptoms, are milder than in the latter, and recovery follows as a rule within a few days.

At onset the fever is about 38-39 C., not infrequently higher, the stools are bloody and mixed with mucus and pus; on the day of onset or the following day there may be 10 stools or so, but on the 2nd or 3rd day the temperature falls and stools are less frequent; 2-3 days later there are only 2 or 3 stools. In most cases in 7-10 days the stool is almost normal or the patient may even be constipated. On palpation of the abdomen one sometimes feels, as in dysentery, cord-like thickening, but this is often absent. Tenesmus is also usually insignificant, because the intestinal inflammation is mild in character.

The symptoms just described are those seen in the greater number of cases, but in more than 15% of all cases on the day of onset there are severe symptoms of intoxication, that is, high fever, drowsiness, cramps, coma, vomiting, weak and frequent pulse, coffee grounds hemorrhagic vomit, marked acetonuria, etc., and most of these become fulminant and end fatally in 2-3 days after onset. Cases are known in which patients, who on the day of onset have shown no special symptoms of intoxication, on the 3rd or 4th day show signs of severe intoxication (vomiting, heart weakness, frequent pulse, coffee ground vomit, jactitation, etc.) and in a few days they die. The latter form is less frequent than the fulminating. The fulminating symptoms of all these cases resemble those of the bacillary dysentery in children. But in paradysentery, the disappearance of the intoxication is followed by rapid convalescence, for the intestinal symptoms are less severe than in true dysentery.

*Infectivity.*—Members of the same family may become ill simultaneously, or at an interval of a few days. It happened that a mother nursing her child became infected with the same type of organism, thus demonstrating that these organisms possess infectivity, similar to other pathogenic bacteria.

*Anatomic Changes in the Intestinal Wall.*—Since 1913, when this group of bacilli was first demonstrated, I have made 6 postmortem examinations—3 persons who died within 36 to 60 hours after the onset of illness, and 2 on patients dying on the 5th and 14th day,

respectively. We may include another case in which an improvement of the dysenteric symptoms took place, but the patient died on the 23rd day from septicemia. It was possible to isolate the causative agent from the intestinal tissues in all these cases except the last.

The following changes were observed in the intestines after death with fulminating symptoms: There was swelling of the lymphatics of the small intestine, especially below the ileum—a particularly characteristic change. The mucosa itself showed no special alterations except slight edema and hyperemia, the latter being, however, more marked on the wall of the large intestine, the follicles of which were swollen and showed erosions or slight ulcers. There were indications of a desquamation of epithelial cells, and occasionally one found a fine, branny coating which microscopically was shown to consist of loosened epithelial and blood cells, etc. These changes are similar to those found in true dysentery, except that in the latter disease the inflammation of the mucosa of the large intestine is far more marked. The indication of manifest diphtheric membrane, such as occurs in true dysentery, could not be found in fulminating cases. The changes found in the small intestine did not differ from those of dysentery.

The necropsies on patients ill for more than 5 days showed the so-called diphtheric inflammation of the large intestine, although not as marked as in dysentery; the lower part of the ileum occasionally showed slight diphtheric inflammation. In short, while the changes of the intestinal mucosa in paradysentery infection resemble those of dysentery, they are slighter in degree.

The other viscera showed no marked changes except parenchymatous degeneration, hyperemia, etc. Occasionally there were a circular, duodenal ulcer, and erosions or small ulcers on the mucosa of the stomach. In fulminating cases the thymus not seldom seemed enlarged.

#### TERMINOLOGY AND CLASSIFICATION

As already stated, bacteriologically as well as pathologically, i. e., as indicated by the clinical phenomena and the changes of the intestinal wall, these bacilli are related to the true dysentery types, the only difference being that the changes caused by them are less severe in character than are those of true dysentery. The organisms are not, however, identical with the dysentery bacilli and for that reason I have employed the term *Bacillus paradysenteriae*, A and B, and called the disease caused by them, paradysentery. It is rational and practicable to separate dysentery and paradysentery according to the causative agents, as we do in the case of typhoid and paratyphoid, although the

two diseases in each case have certain clinical and anatomic resemblances. In English, American and German literature the so-called acid strains of dysentery bacilli are referred to sometimes as paradysentery bacilli, but as it is desirable that all types of the dysentery bacillus which possesses in general similar biologic properties should be regarded as constituting a single group, as emphasized by Shiga in Japan,<sup>5</sup> Lentz in Germany,<sup>6</sup> and Hiss in America.<sup>7</sup>

#### COMPARISON OF DYSENTERY AND PARADYSENTERY

A comparative study of cases yielding the dysentery and paradysentery organisms observed for a 4-year period (1916-1919, inclusive), during which particular care was exercised in the isolation of the paradysentery organisms, comprises 195 cases of the former and 65 of the latter, or a ratio of 3 : 1. As already stated, the patients suffering from paradysentery in general show less marked symptoms, so that they are likely to come under our treatment less frequently than those suffering from dysentery. Hence it would seem that the ratio given is not absolutely correct and that the proportion of cases of paradysentery is probably larger than here indicated.

The children suffering from fulminating symptoms of paradysentery range in ages from 2 to 6 years, and the course of the disease is similar to that of dysentery of the fulminating type. However, such fulminating symptoms are not infrequently present after orthopedic operations for congenital malformations, such as talipes equinovarus, congenital articular paralysis, etc., and some times such fulminating symptoms develop without the presence of any kind of pathogenic organism. According to our observations, these symptoms in dysentery and paradysentery are referable to the constitutional disposition of the patient rather than to the toxic action of the bacteria.

#### PARATYPHOID-X BACILLUS

As already stated, the organisms isolated by the writer from cases showing dysenteric symptoms were, as a rule, *B. dysenteriae* and *B. paradysenteriae*. But in a number of cases I was able to demonstrate true paratyphoid B and the paratyphoid X bacilli (Mita). I shall now describe the general properties of the paratyphoid X bacillus.

The bacilli may be cultivated abundantly by isolating them from the mucoid stools in the early stages of the disease. Colonies grown on an agar medium are spherical and resemble those of the dysentery

<sup>5</sup> Clinical Bacteriology and Infectious Diseases (Japanese), 1914.

<sup>6</sup> Kolle and Wassermann, Handb. d. path. Mikroorganismen, 1914, 3, p. 905.

<sup>7</sup> Hiss and Zinsser, Textbook of Bacteriology, 1917, p. 435.

and paratyphoid bacilli. The bacilli, which are of the same size as *B. coli*, at times show threadlike connections and stain with various anilin dyes; they are gram-negative. It is difficult to say whether or not they have flagella. Although some of them seemed to me to have a feeble active motility, none has been seen to move across the field of vision. Most of them show molecular activity.

*Indol*.—Our paratyphoid bacilli do not form indol even after remaining for a long time in from 1 to 2% Witte peptone solution.

*Milk*.—They do not coagulate milk or cause it to separate, even after long cultivation.

*Litmus Whey*.—After 24 hours the bacilli stain litmus whey a fast violet, occasionally a moderate red followed by violet.

*Witte Peptone Solution*.—There is no scum formation after long continued cultivation in Witte peptone; the solution becomes slightly cloudy.

*Gelatine Stab Cultures*.—These show good growth along the line of the stabs; there is no liquefaction.

*Neutral Red Glucose Agar Stabs*.—These show feeble gas formation and fluorescence.

*Carbohydrate Decomposition*.—In 1 to 2% Witte peptone solution, containing respectively 1.3% of mannite, dextrose, saccharose, maltose, dextrin, or lactose, the mannite and dextrose were converted into acids and gases, but the other sugars failed to decompose. The bacilli differ in this reaction from paratyphoid A and B, as dysentery 2 (Y) differ from type 4 (Flexner).<sup>8</sup>

*Differentiation*.—The paratyphoid X bacilli will be agglutinated by the serum of the patients from whom they are isolated, as well as the serum of the immunized rabbits. Agglutination takes place in a dilution of 160:320.

The extract of the bacilli produces complement deviation with the patients' serum and immune rabbit serum.

Paratyphoid X bacillus isolated for the first time in 1917, and another strain possessing the same characteristics was isolated in 1919. The two strains show reciprocal agglutination and complement deviation. The serum of patients yielding the organisms failed to agglutinate other types of bacilli, nor does the serum of rabbits immunized with these bacilli agglutinate the latter. Furthermore, the X bacilli show no agglutination reaction with the serum produced by injecting other bacilli.

<sup>8</sup> Kolle and Wassermann: Handbuch. d. path. Mikroorganismen, 1914, 3, p. 1005.

TABLE 1  
DIFFERENTIATION OF THE TRUE DYSENTERY, PARADYSENTERY (MITA) AND PARATYPHOID  
BACILLI GROUPS

Group	Type	Motility	Liquefaction of Gelatin	Indol Production	Litmus Whey	Milk Coagulation	Fermentation of Carbohydrate					Remarks	
							Mannite	Dex-trose	Saccha-rose	Mal-tose	Dex-trin		Lac-tose
The true dysentery bacilli group	Type 1	Molecular	—	—	* later 1 or or 1 later —	—	—	*	—	—	—	—	Sluga type
	Type 2	Molecular	—	+	* later 1 or or 1 later —	—	*	*	—	— or — later *	—	—	Y type
	Type 3	Molecular	—	+	* later 1 or or 1 later —	—	*	*	— later *	— or — later *	—	—	Strong type
	Type 4	Molecular	—	+	* later 1 or or 1 later —	—	*	*	— later * or — later —	*	*	—	Flexner type
	Type 5	Molecular	—	+	* later 1 or or 1 later —	—	—	*	— later —	*	*	—	—
Paradysentery bacilli group according to Mita	Type A	Molecular	—	—	* later 1 later * or or 1 later — later *	— later +	*	*	— later *	*	*	— later *	Like type 4†
	Type B	Molecular	—	—	* later 1 later * or or 1 later — later *	— later +	*	*	— later *	— later *	— later *	— later *	Like type 2‡
Paratyphoid bacilli group	Type X (Mita)	Slight motile?	—	—	* later 1 or or 1	—	+	+	—	—	—	—	Like type 2‡
	Type A	Actively motile	—	—	* later 1 or or 1	—	+	+	—	+	*	—	Like type 4; †
	Type B	Actively motile	—	—	1 later —	— later clear	+	+	—	+	+	—	Like type 4; †

† Of dysentery bacilli during beginning of fermentation.

‡ Of dysentery bacilli unless gas formation.

+ = positive, acid and gas, or coagulation of milk.

— = negative, no change, or alkaline.

\* = acid, but no gas.

I = slight acid.

CLINICAL OBSERVATIONS OF PATIENTS YIELDING THE  
X BACILLUS

The bacilli isolated in 1917 were obtained from a child evacuating dysenteric stools containing mucus and traces of blood. The patient, a 2-year old girl, who died 2 weeks after the onset of the disease, also showed typhoid symptoms, i. e., continuous fever and the evacuation of 2 or 3 mucoid stools daily.

The same bacilli were isolated in 1919 from 2 cases, a brother 4 years old and a sister 6 years old, who were taken ill at the same time. These patients had general and intestinal symptoms not readily distinguishable from mild dysentery. The temperature was about 38 C. on the first day when there were 8 or 9 mucoid stools mixed with blood and pus, but on the following day the stools diminished to 1 or 2, and the temperature fell; after a week constipation set in. As already stated, these bacilli were isolated from only a few patients, in one case from a patient suffering from so-called typhoid symptoms, and in 2 others from patients showing symptoms resembling those of mild dysentery.

## NAME AND CLASSIFICATION OF PARATYPHOID X

While the organisms were isolated from patients showing dysenteric symptoms and also those showing typhoid symptoms, in their characteristic of changing mannite and dextrose into acids and gases, they were analogous to the paratyphoid group. For the reasons cited, I have classed the bacilli in question with the paratyphoid group, and have named them "paratyphoid X bacilli."

## SUMMARY

Various types of bacilli may be the causative agents of dysentery and dysenteric affections in children. While as a rule the dysentery bacilli are isolated, not infrequently the other kinds of bacilli are present. On the basis of these investigations it seems that at least one fourth of the cases are traceable to organisms not of the true dysentery type. Of the organisms producing dysenteric symptoms, aside from the true dysentery bacillus, in the majority of cases the paradysentery bacillus now described is found.

The paradysentery bacilli are small rods having no motion of their own, and forming acids by the fermentation of glucose but no gases. Two or three weeks after cultivation, milk begins to coagulate from the bottom up, and coagulation is completed with a few days. Older

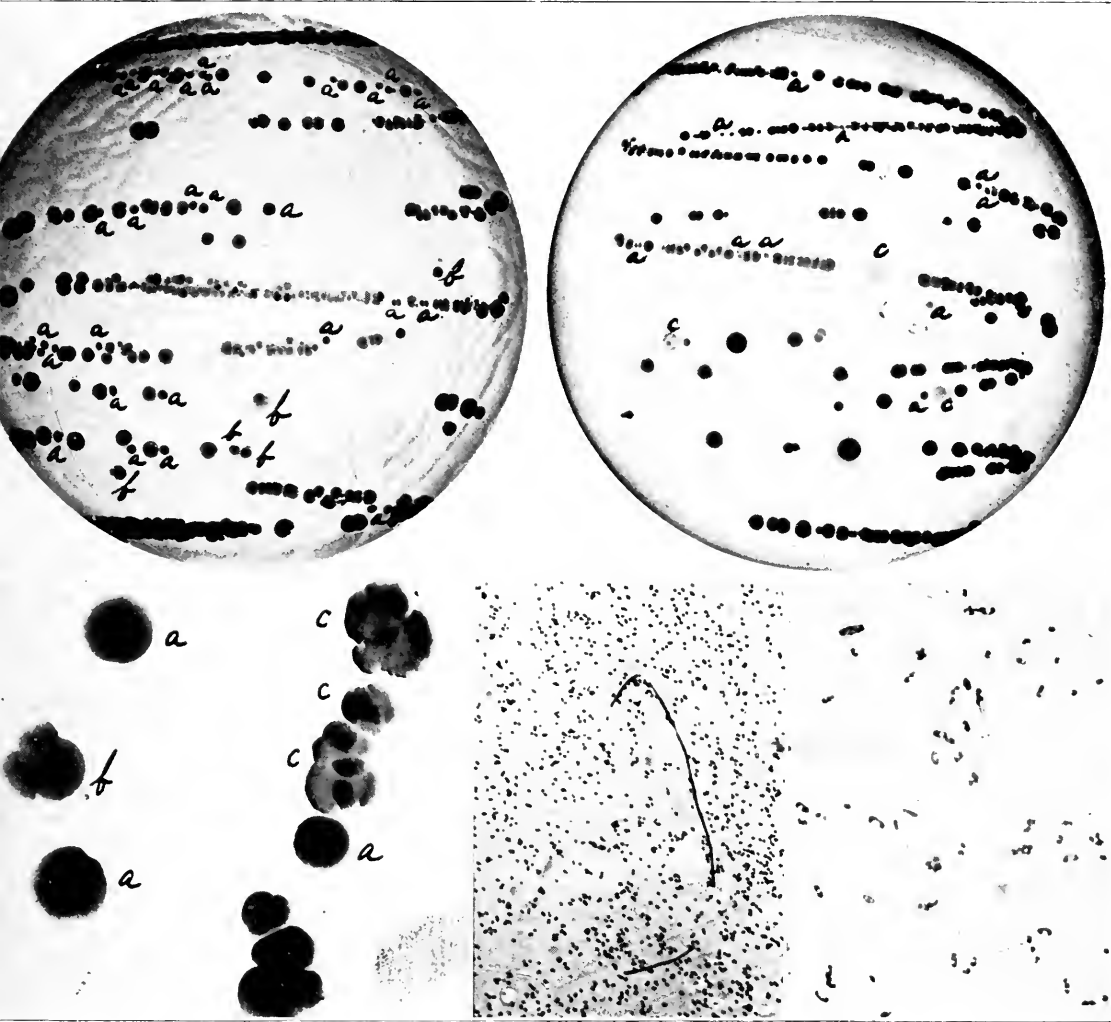
strains will coagulate milk even sooner. In addition to the characteristic round colonies, our paradysentery bacilli form also irregular colonies. The paradysentery group may be divided into two types, A and B, according to the carbohydrate decomposition produced. The organisms are infectious and pathogenic in character, and produce slight diphtheric inflammation on the intestinal wall.

The disease caused by paradysentery bacilli group should be called paradysentery.

In addition to the bacteria mentioned, the symptoms of dysentery may occasionally be produced also by paratyphoid or similar organisms. One group consists of rodlike organisms possessing feeble motility (?) which form acid and gas from mannite and dextrose, but do not affect saccharose, maltose, dextrin and lactose. These organisms, when attacking man, produce slight dysentric or typhoid symptoms. The organism is called "Paratyphoid X bacillus."



PLATE 1



Figs. 1 and 2.—Colonies of paradysentery bacilli, isolated from the feces. Plain agar plate, natural size; a, round colonies; b, lobulated colonies; c, irregular colonies. Those not marked are colon bacilli.

Fig. 3.—Irregular colonies developing from a pure culture of the paradysentery bacillus. The changes in the shape of the colony do not indicate any change in the biologic properties of the organism.

Fig. 4.—Long chains of paradysentery bacilli, a feature of the organism which distinguishes it from the organisms of the dysentery group.

Fig. 5.—Bipolar staining of paradysentery bacilli; Ziehl's solution.



# INDUCED MORPHOLOGIC VARIATION IN *B. COLI*

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In recent years there have appeared from time to time reports of marked morphologic changes occurring in cultures of bacteria when kept on various mediums at different temperatures. The authors of these reports have in some cases called them merely morphologic changes due to environment; in other cases the different morphologic responses were interpreted as a complex process for the perpetuation of the species. It was for the purpose of casting a little more light on this subject that the present work was undertaken.

## HISTORICAL REVIEW

The literature on the subject of this report may be briefly summarized by a description of the results obtained in the most important investigations of this character.

Almquist,<sup>1</sup> in 1893, reported that *B. coli* communior when grown in sterile sand moistened with sterile manure extract and incubated at low temperatures produced polar granules which when freed formed a new growth. When the polar granules remained attached to the cell wall they grew as coccoids.

Haslam<sup>2</sup> by the use of mediums containing high percentages of nitrogenous or carbohydrate material, obtained from a strain of *B. coli* both large rods and coccoidal forms. Cultures of both forms even after five months on these mediums reverted to the normal type when planted on standard mediums.

Adami et al.<sup>3</sup> found that in body fluids *B. coli* produced stumpy bacillary forms with round chromatin bodies, like cocci, at the poles, or else large filamentous forms which broke up into the component stumpy bacilli. Abott,<sup>4</sup> continuing this work, reported that the minute diplococcoid forms when cultivated for some time on ascitic fluid became fixed so that they did not revert wholly to type.

Ohlmacher,<sup>5</sup> in working with a culture of *B. coli*, obtained from early autopsy, found that it produced on agar plates all gradations from diplococcoid and small streptococcoid-like individuals to long coarse filaments. Four transfers through bouillon restored it to the usual morphology. It gave the characteristic physiologic reactions, while still irregular in morphology.

Walker and Murray,<sup>6</sup> in testing the effect of vital staining on bacteria, discovered that *B. coli* produced long filaments which sometimes looked like a chain

Received for Publication, July 18, 1921.

<sup>1</sup> Ztschr. f. Hyg. u. Infektionskr. 1893, 15, p. 283.

<sup>2</sup> Jour. Path. & Bacteriol., 1892, 5, p. 189.

<sup>3</sup> Jour. Exper. Med., 1899, 4, p. 349.

<sup>4</sup> Jour. Path. & Bacteriol., 1900, 6, p. 326.

<sup>5</sup> Jour. Med. Research, 1902, 7, p. 128.

<sup>6</sup> Brit. Med. Jour., 1904, 2, p. 16.

of cocci when grown on a medium containing 0.2% of a saturated alcoholic solution of methyl violet. These organisms quickly regained their normal form on standard mediums.

Wilson,<sup>7</sup> employing a Conradi Drigalski medium containing urea, obtained thread-like forms with round or oval swellings. These swellings occurred most abundantly when the inoculum was taken from a comparatively old agar culture. The bulb-like growths were also found on the ends of the short lateral branches; they sometimes occurred free or with the remnant of a filament attached. Bacilli were found budding off from the poles of the threads or from the termination of the lateral branches. Coccoidal forms also appeared to be derived by budding from the tips of the lateral branches. These lengthened out into rod-like segments. The filaments did not persist in subcultures on standard mediums.

Hata<sup>8</sup> obtained thread-like organisms and globular bodies on mediums containing magnesium, calcium or sodium chloride.

Revis,<sup>9</sup> by growing his cultures in a broth containing increasing quantities of malachite-green up to 0.1%, discovered that two out of four of them lost the power to produce gas. This function could not be restored by repeated transfer to a medium containing sugar. Microscopic examination revealed the presence of long filaments in the culture. The growth thus produced from the typical *B. coli* was neither physiologically, morphologically nor culturally a colon bacillus.

Löhnis and Smith<sup>10</sup> made a comparative study of 42 strains<sup>11</sup> of bacteria by employing various mediums and making frequent microscopic examinations of both young and old cultures. They found that all the bacteria studied lived alternately in an organized and in an amorphous (sympylastic) stage. "Regenerative units" appeared in the amorphous material and by germination or stretching became cells of normal shape. There was a direct union (conjunction) between two or more cells. All the bacteria multiplied not only by fission, but also by the formation of gonidia. Some of the gonidia were filtrable. The life cycle of each species of bacteria was composed of several subcycles showing wide morphologic and physiologic differences.

Almquist,<sup>12</sup> in his latest publication, reports that he obtained filaments and coccoidal bodies by growing 2 organisms similar to *B. coli* on old dry agar for 11 days, at 14 C. At 35 C., one of the cultures produced lightly and darkly stained rods, gonidia and rods in conjunction.

Hort,<sup>13</sup> in a further study of the morphologic variation of bacteria found, by observing the growth of a single organism on the warm stage, that binary transverse fission eventually held the field, though not absolutely, to the exclusion of other kinds of reproduction. Budding (gemmation) occurred freely in conjunction with ordinary binary fission until colonies began to form on the solid medium. This investigator<sup>14</sup> concludes from his latest work that the deeply staining organisms found in some cultures are types of resting cells which are able to perpetuate the strain when the environment becomes unfavorable; they

<sup>7</sup> Jour. Path. & Bacteriol., 1904, 11, p. 394.

<sup>8</sup> Centralbl. f. Bakteriöl. I. O., 1908, 46, p. 289.

<sup>9</sup> Proc. Roy. Soc., B, 1912, 85, p. 192.

<sup>10</sup> Jour. Agric. Research, 1916, 6, p. 675.

<sup>11</sup> *B. coli* was not among the species studied.

<sup>12</sup> Svenska Läkaresällskapets Handlingar, 1917, 43, p. 543.

<sup>13</sup> Proc. Roy. Soc., B, 1917, 89, p. 468.

<sup>14</sup> Jour. Hyg., 1920, 18, p. 269.

increase in number with the age of the culture but cannot be produced by any given cultural conditions. Some cultures that do not contain these cells exhibit a lower viability than those that do.

## EXPERIMENTAL WORK

*Source and Treatment of Cultures.*—In order to study the morphologic variation of *B. coli* in different environments 28 cultures of this species of bacteria were obtained from different laboratories in various parts of the country.

TABLE 1  
LABORATORY NUMBERS OF ORGANISMS, NAMES GIVEN BY THE BACTERIOLOGIST WHO SENT THEM, THEIR SOURCE AND DATE OF ISOLATION

Number	Name	Source	Isolated
1	<i>B. coli</i> .....	Brownsville dysentery epidemic.....	1911
2	<i>B. coli</i> .....	Normal human feces.....	1910
3	<i>B. coli</i> .....	Medicinal spring water.....	1910
4	<i>B. coli</i> .....	From a bird.....	1901
5	<i>B. coli</i> .....	Bovine feces	
6	<i>B. coli</i> .....	Bovine feces	
7	<i>B. coli</i> .....	Human feces, D. C.	
8	<i>B. coli</i> .....	Human feces, D. C.	
9	<i>B. coli</i> .....	Human feces, Pennsylvania	
10	<i>B. coli</i> .....	Pasteur Institute	
11	<i>B. coli</i> .....	Mineral water, California	
11a	<i>B. coli</i> .....	Mineral water, California	
12	<i>B. coli communior</i> .....	Human feces	
13	<i>B. coli</i> .....	From a pigeon	
14	<i>B. coli</i> .....	From a hen	
15	<i>B. coli</i> .....	From a turtle	
16	<i>B. coli communis</i> .....	Human feces	
17	<i>B. coli com.</i> .....	Unknown.....	1916
18	<i>B. coli</i> .....	Bovine from intestinal feces.....	1916
19	<i>B. coli</i> .....	Bovine from intestinal feces.....	1916
20	<i>B. coli</i> .....	Human feces.....	1914
21	<i>B. coli</i> .....	From a case of cystitis.....	1911
22	<i>B. coli</i> .....	Milk, Illinois.....	1913
23	<i>B. coli</i> .....	Codfish feces	
24	<i>B. coli</i> .....	Pigeon feces	
25	<i>B. coli</i> .....	Y. M. C. A. swimming pool	
26	<i>B. coli</i> .....	Human feces	
27	<i>B. coli</i> .....	Oysters, Jamaica Bay, N. Y.	
28	<i>B. coli</i> .....	Soil at Veronica Spring, Calif.	

When these organisms were received they were carefully plated on beef agar and after 2 days' growth at 37 C. an inoculum was taken from the typical colon-like colonies and planted on beef agar slants. The cultures were next planted in broth and later plated on gelatin. The growth on these plates was employed as the inoculum for duplicate beef agar slants, one typical colony of each strain being selected for this purpose. These cultures were incubated for 2 days at 28 C. when one set was placed in the refrigerator for stock and the other one employed for the laboratory tests. Fresh duplicate slants were made from time to time from the stock cultures; one of the duplicates always being kept in the refrigerator for stock.

When taken from day old cultures on beef agar all except 3 of the strains were found to consist of the short rods with rounded poles that are characteristic of typical *B. coli*. One of these 3 contained small and medium sized rods and some threads and the other 2 were almost coccoidal in form. They all took the ordinary stains readily. All were gram-negative. None of them gave any evidence of spore formation. In day old cultures in beef broth 23 strains were motile and 6 non-motile.

One day old cultures were planted in glucose, lactose, saccharose and mannite broths. Two cultures, 4 and 8, that failed to produce gas in the glucose and lactose broths, were retained. They were tested with the *B. coli* in order to ascertain whether their reaction to the various mediums would show any marked differences. Eleven cultures produced gas in saccharose. The indol test showed 20 positive and 9 negative. In the duplicate tubes for nitrate reduction, 4 organisms produced a small quantity of nitrate in one set and none in the other; the remainder were good positives. The test for ammonia gave the same number of negative results. As the latter tests are not specific, they were not repeated. All except 2 cultures, 17 and 26, acidified and coagulated milk after 3 days at 37 C. Gelatine was not liquified by any of the cultures after 3 weeks' incubation at 20 C. Day old cultures on beef agar were also inoculated into Clark and Lubs' synthetic phthalate-glucose-phosphate solution for the determination of the Voges-Proskauer and the methyl red reactions. The cultures were incubated at 30 C. for 2 days and 5 days. The 2 day old cultures were used for the determination of the Voges-Proskauer reaction, and all were negative except two (22 and 23). These *B. aerogenes* strains were retained and tested with the *coli* cultures. The 5 day old cultures were employed for the methyl red test. The *B. coli* strains gave a  $P_H$  varying from 5.3 to 5.6. These different tests showed that 23 of the cultures were typical *B. coli* strains, while the remaining 6 were not. The morphology of some of the strains may be seen in fields 1 to 5 inclusive in plate 1. The rods of culture 13, field 2, lack the plumpness characteristic of *B. coli* cells. This strain, however, gives all the typical reactions of the species.

*Methods and Mediums.*—When this investigation was started a great variety of mediums were employed in order to produce a change in morphology. The cultures were found to be remarkably constant. Changes in morphology were obtained by employing unusual mediums, such as whole egg slants, egg agar, glucose egg agar, egg ammonium

lactate agar and egg starch agar, but in no case did more than 2 strains at the same temperature give the same morphologic response to a medium. As a case in point the variations with egg starch agar may be cited. Two cultures when grown for one day on this medium at 2 temperatures produced at 17 C. mostly short to medium rods, fields 8a and 9a, plate A; at 37 C. the organisms were smaller and consisted of very short rods, ovals and coccoidal forms, fields 8b and 9b, plate 1. The morphology was also studied in old cultures, but the results were not much more satisfactory in this case than when the young cultures were employed. A careful search of the stained films made from the young cultures revealed a few gonidia but, with the exception of one culture, the number found were very few.

As considerable difficulty was experienced in producing a uniformly smooth slant of whole egg after sterilization the method finally adopted is described for the benefit of those who may wish to use this or a medium producing similar difficulties. The eggs, which must be fresh, are strained 4 times through 4 thicknesses of cheesecloth. They are poured into a small suction flask and placed under a vacuum for 5 minutes with occasional shaking. About 0.75 cc is run into tubes (4" x 1/2") with a pipet and the egg then coagulated by placing the tube in a slanting position in boiling water or in an inspissator. When it is coagulated the egg is just covered with cold, distilled, sterile water and the tubes plugged and placed in a beaker containing cold water. They are sterilized in the autoclave for 30 minutes at 16 lbs. of pressure. The water over the slants may be poured off just before using. When treated in this way they will undergo this long sterilization and keep indefinitely without drying out.

When immersed in a beaker or pan of water tubes of milk may also be sterilized in the autoclave.

## PLAIN EGG AGAR

Whole egg.....	2 cc
Agar 1.5%.....	7 cc

Fresh eggs must be used. Strain the eggs twice through four thicknesses of cheesecloth. Mix well with agar. Sterilize at 12 lbs. for 15 minutes on a slant stick or glass rod.

## GLUCOSE EGG AGAR

Whole egg.....	2 cc
Agar 1.5% } .....	7 cc
Glucose 1.0% }	

See directions for plain egg agar.

## AMMONIUM LACTATE AGAR

Na <sub>2</sub> HPO <sub>4</sub> .....	2.0 gm.
Agar .....	15.0 gm.
Distilled water.....	1000 cc
Titrate to plus 3. Then add	
NH <sub>4</sub> C <sub>2</sub> H <sub>3</sub> O <sub>2</sub> .....	10 cc
CaCO <sub>3</sub> .....	10 gm.

Prepare the egg medium by adding 2 cc of whole egg, carefully strained, to 7 cc of this medium. Tube and sterilize at 12 lbs. of pressure for 15 minutes.

## EGG STARCH AGAR

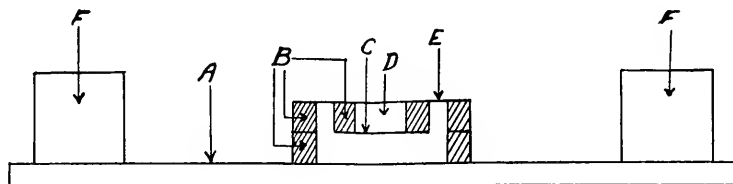
The egg starch agar is made from whole egg prepared as described in the foregoing and then 2 c c of this product are added to 7 c c of a medium containing 1.5% agar in distilled water and 0.1% soluble starch. It is slanted and sterilized in the same way as the plain egg agar.

*Microscopic Study of the Living Organisms.*—As the regular cultural methods produced no satisfactory results, a microscopic study of the living organisms was undertaken.

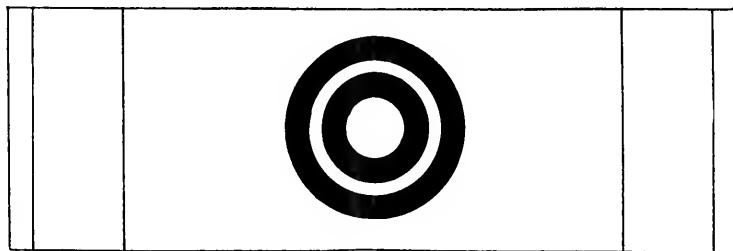
A cell was perfected in which these bacteria could grow satisfactorily and be kept under observation for a long time. It was prepared by cutting 2 rings of mechanical sheet packing about 20 mm. in outer diameter and 15 mm. in inner diameter. The packing material contained Para rubber, plantation rubber (smoked sheet) whiting, asbestos pulp, barytes, litharge, black lead talc and sulphur. Cork cutters were employed for the cutting of rings. The material used in this investigation was 1 mm. thick. One of these rings was fixed to the middle of the slide with Canada balsam and the second one then secured on top of it with the same adhesive. A third ring with an outer diameter of 13 mm. and an inner one of 9 mm. was then concentrically fastened to a clean cover glass of about the same diameter, 20 mm., as the outer edge of the rings on the slide; fig. 1 shows the structure of the cell. A is the slide, B the rings of mechanical sheet packing, C the small cover glass, D the small sealed cell, E the large cover glass and F the plasticine supports.

The slides and cover glasses were not used until the following day so that the balsam would have time to set. When cultures were to be made the cover glass with its ring was first sterilized by passing through a flame and then setting it in a sterile Petri dish. The slide with its rings was sterilized in the same way and put in the same Petri dish. A small quantity of sterile petrolatum was then spread around each ring with a sterile scalpel. The culture was prepared by smearing the inoculum on the cover glass within the ring, adding 2 or 3 drops of sterile broth from a pipet and then pressing down on the ring a small sterile cover glass of about the same diameter as the ring. There was thus formed a small sealed cell completely filled with solution. The larger cell on the slide was filled with sterile solution and the cover glass with its closed cell pressed into position. A double cell was made so that it would form a better protection against loss of solution. A strip of plasticine about 2 mm. higher than the cell was placed at each end of the slide so that when it was inverted and allowed to rest on





*CROSS SECTION*



*PLAN*

Fig. 1.—Structure of cell.



the plasticine the cell would be well clear of the surface below. The slide was incubated in a sterile Petri dish in an inverted position so that the organisms that settled from the solution would rest on the cover glass and be easily examined microscopically.

A modification of this type of cell that was of service was one in which the ring fastened to the cover glass consisted of one thickness of waxed paper. The advantage of this cell was that with the oil immersion the observer could look through the whole depth of the solution. The entire culture with its organisms could thus be examined. This cell was only serviceable when there was no gas production for the pressure of gas invariably forced the inner cover glass off.

The medium that gave the most favorable results with *B. coli* was an ammonium citrate broth made as follows:

K H <sub>2</sub> PO <sub>4</sub> (neutral to phenolphthalein with $\frac{n}{20}$ NaOH.....	0.2 gm.
NaCl .....	0.2 gm.
MgSO <sub>4</sub> .....	0.2 gm.
CaCO <sub>3</sub> .....	excess
FeCl <sub>3</sub> .....	trace
Ammonium citrate .....	1.0 gm.
Glucose .....	1.5 gm.
Distilled water .....	1000 cc
Filter clear. Tube and autoclave at 15 lbs. of pressure for 15 minutes.	

Many cultures were examined in this cell but 10 was the only one that gave results of particular interest. It produces gonidia regularly on all mediums and more abundantly in 1-3 day old cultures than in older ones. These bodies, which are nearly always polar, are at first visible as a minute spherical cell separated from the parent by a constriction in the cell wall. They grow in this close connection until they have attained the diameter of the mother cell when the constriction is gradually elongated, principally, it would seem, by the active movement of the small cell. The constriction may attain a length of 1 micron. The whiplike snapping movements of the polar body finally break the connection, and the gonidium becomes an actively motile cell. The gonidium is much more active than the large organisms and on account of the rapidity of its movement and small size there was no opportunity to make a continuous observation of its growth after separation from the parent cell.

Indirect evidence of the growth of gonidia was obtained from the general appearance of these cultures and also aerobic ones which, during

the first 3 days, usually show a considerable number of gonidia, but as the cultures age the number of gonidia becomes less and oval forms and short rods more numerous.

A contact preparation from a colony on beef agar, field 24, plate 2, shows organisms with polar gonidia; some of the rods are almost wholly divided into small living units. The culture that produces gonidia most abundantly also usually contains more rods sticking together than the other cultures do. This adhesion of two or more cells Lohnis and Smith<sup>10</sup> have called conjunction. Fields 10 and 13, plate 1, show cells so joined.

When this culture, 10, is planted in an ammonium citrate broth in the cell described and incubated at 37 C., there is at first only the normal growth, but after 6 or 7 days a marked difference in the cells becomes evident. The gonidia on the cover glass\* or the buds still attached to the mother cell are, after this time, an opaque black while the rod remains vitreous. Some of the very short, thick rods in this culture may also become entirely black, but the long, thin rods characteristically occur either with black granular contents or with no granules. The granules are not found in any regular arrangement in the different rods. They first appear brown and finally black. This blackening was produced by the chromatin absorbing some substance which was dissolved out of the packing material. This substance occurred in a pale yellow fluid, which spread over the cover glass, and it affected only those organisms that settled in the field. The bacteria with black granules did not occur in the solution deeper than 32 microns from the cover glass. In order to determine whether this blackening of the gonidia and chromatin granules could be traced to the presence of any one particular constituent of the packing material each of its ingredients was tested in a glass cell, but in no instance did the organisms give a positive reaction.

The different absorptive power of a gonidium and a mother cell indicates that when a gonidium is formed the process is not similar to fission, except possibly in very young cells, but that there is protoplasmic differentiation whereby a nucleus is formed for the gonidium, either by the growth or the swarming of nuclear material. The important fact is that there is a difference in the contents of the two cells in that the more active nuclear material seems to fill completely the younger cell. The accumulation of the black material in the cells is a gradual process and is a measure of the activity of the cells. It

eventually results in their death. The blackening of the small cells cannot be attributed to the permeability of the cell wall because the granules in the long rods become black like the gonidia.

The black nuclei were often found near the poles of the large rods. If there was a black bud attached to the pole there was usually no black nucleus at that pole, but there was frequently one at the opposite pole. In only one case was a black nucleus found near the pole when there was still a bud attached to it. The evidence gained from an examination of many slides containing cultures of this particular organism indicated that the nuclear material, increasing slightly in size, is gradually moved to the pole of the rod; the constriction process, by which it is finally separated from the parent cell as a gonidium, then begins.

The organisms in the other cultures were smaller and so were more difficult to examine. They showed some black cells and a very few gonidia.

While these methods of studying the morphologic changes of *B. coli* had yielded something of value for certain strains, they had, as a whole, failed because of the lack of uniformity of response by the cultures. For this reason it seemed advisable to try the influence of osmotic pressure on the bacterial cells.

*Experiments with a Medium of High Solution Pressure.*—The medium first employed for these tests was a standard beef agar containing from 2% to 10% of sodium chloride. The agar was selected in preference to the broth because in making a film for microscopic examination less foreign material is carried from the agar than from the broth.

All the strains grew well on the agar with a salt content up to 5%; above this amount the growth was less abundant, and at 8% all except 3 failed to grow. The majority of the strains showed a change in morphology.

When these preliminary tests were completed the cultures were plated on beef agar and duplicate beef agar slants were then inoculated from the typical colonies that appeared on the plates. The cultures were grown for one day at 37 C. and then inoculated into Clark and Lubs' synthetic phthalate-glucose phosphate solution for the determination of the Voges-Proskauer reaction and the methyl red test. The results were the same as those first obtained.

The pure cultures from this last plating were grown on both fresh and old salt agar and examined microscopically. The organisms from

old somewhat dry salt agar showed a slightly greater morphologic variation than the organisms on fresh salt mediums. In order that new mediums might be made more nearly comparable to the old dry one, which had given the best results, the amount of agar in them was increased to 2.5%. The mediums thus prepared showed no material change in  $P_H$  value from the standard one which gave a reading of 6.9 against that of 6.8 for those containing 5, 6 and 7% salt.

When grown on the beef agar containing 2.5 agar and 6% salt the cultures produced two characteristic types of growth. One gave a scanty to moderate filiform, raised, glistening, smooth, opaque, grayish white, slimy growth; the other gave a very scant filiform, flat, dull, granular, translucent, colorless, brittle growth. The 3 organisms that grew on agar containing 8% salt, belonged to the former group. A heavy inoculum on the salt agar slant usually produced a growth; a light one frequently failed to do so. Growth from the heavy inoculum started where the smear was thickest and the thin portion appeared to dry out. From this evidence it seems as if mass action may play a part in enabling the organisms to grow on this medium. There is also a possibility that growth may be due to the multiplication of the resistant organisms described by Hort.<sup>13</sup>

With this preliminary statement about the growth of *B. coli* on salted beef agar the morphologic variation induced by this medium may now be considered.

*Results of Microscopic Examination.*—Before considering the results of the microscopic examination of the strains, as shown by the accompanying photomicrographs, it must be clearly understood that the organisms in a culture do not all produce the same morphologic type on a medium. There is usually considerable variation in a single culture and also in repeated cultures as four transfers of the strains from beef agar to salted agar have shown. Three of the strains were very stable, little or no variation being evident; the remaining strains produced morphologic types that have been grouped under the following headings: gonidia, buds, coccoidal types, branched types, segmentation and threads. A brief explanation of these terms will be given when necessary under the respective headings.

*Gonidia:* Gonidia are the small, usually actively motile spherical or oval organisms which first appear on bacterial cells as buds, but which later, due possibly to their own active movements, become free. The organisms shown in the various fields on plate 2, under the designation

of gonidia, are in reality mature buds that are near the stage when they will be liberated as gonidia. The buds are shown in order to leave no doubt in the reader's mind concerning the identity of these minute cells.

A careful examination of all the *B. coli* on plain beef agar showed that on this medium only one culture produced gonidia in sufficient number to be readily found. The picture of this organism has already been referred to—field 24, plate 2. Gonidia may be seen at the poles of each of a number of the rods and threads and in some cases 3 or more of these reproductive units are at the pole of a mother cell. When the *B. coli* cultures were planted on salted beef agar slants they nearly all produced gonidia, but the count of the latter varied from very few to a moderate number, according to the culture. No strain, except 10, has ever produced them in the abundance shown in the field referred to. Fields 22 and 27 inclusive on plate 2, nearly all of different cultures, contain examples of gonidia and mature polar buds that are almost free. Above the thread with the bud at the upper pole in field 22, two slender rods appear to have produced a regenerative body which from its mode of production would be similar to a zygospore. Much importance must not be attached to this type, however, as it was the only example of the kind found. Fields 23a and 23b appear as normal *B. coli*-producing gonidia. In field 25a a branch is dividing from a mother cell; there are buds at both of the poles on the left of this organism. A single gonidium lies below the thread in field 25b; the thread has a mature bud at one pole.

Field 26 shows how differently cells in the same culture respond to the unfavorable environment. At the right of the picture a thread has divided into 4 rods of approximately normal size, while in the middle of the picture a cell has produced 4 short branches from which either rods have been set free by segmentation or gonidia liberated. A mature bud is about to be set free at the upper pole of this cell. Fields 28 to 30 inclusive, plate 2, show mature buds attached to the sides of threads. In the last picture of this group the thread to which the bud is attached is joined to the one below it by a short process. It was impossible to determine whether this was merely slime or whether it actually had some significance.

*Buds:* The term buds is here employed to designate the minute growths that push out from the bacterial cell; they first appear as a slight swelling which may grow into a branch or become a coccoidal cell attached to the parent. Only the beginning of bud formation is shown under this heading because pictures of mature buds have already

been employed to illustrate gonidial growth. Fields 31 to 42 inclusive, plate 2, contain examples of buds in different stages of growth. Nos. 31, 32a and 33a show the bud as a slight swelling in the parent rod; 33b shows a larger growth while in 34 and 35 the bud appears to be forming a coccoidal body at the extremity of the short stem as if it would free a spherical gonidium instead of growing to a rod and then undergoing segmentation. The latter process may have left the short branch remaining on the parent rod in 37, but the branch with the rod still attached may be clearly seen in 75, plate 4, where the branch at the right is dividing. In fields 32b and 42, plate 2, the bud has grown and produced a coccoidal body. A bud that has grown to a short rod and seems to be in the process of separating from the parent cell by sagittal segmentation may be found in the middle of field 80, plate 4. The appearance of some of the smaller rods in this field indicates that they too may have been produced in this manner.

*Coccoidal Types:* Small or large round living bodies of bacterial origin are here classified as coccoidal types. They may be either free or attached to a mother cell. The fields 22, 40 and 43a to 48b inclusive, plate B, and 49 to 68 inclusive, plate 3, all contain examples of coccoidal growth. The long threads produced by some of the organisms in the different cultures show the stimulatory effect of the salt and high osmotic pressure. When the nuclei start to grow in the parent cell, they frequently do so in the form that will best enable them to resist the high solution pressure, this form of course being coccoidal. Fields 22 and 40, plate 2; 49, 50, 51, 56a and 57 to 68, plate 3, all contain good examples of the growth of a nucleus within the mother cell. Field 42, plate 2, contains a much segmented thread; at the pole, where gonidia are usually liberated from the mother cell, the segment has become nearly coccoidal in form. While a nucleus is undoubtedly the source of larger growth in this polar cell as well as in 22, 40, 43a, 45, 47 and 48a, a nucleus is also very likely present in the fourth segment from the one previously mentioned in field 42. This cell is deeply stained like the polar coccoid and it also is larger than the other segments of the thread; again like the coccoid, but unlike the polar segment, it has maintained the rod-like form. In some threads the best types of coccoids are found nearer the middle of the cell (field 45, plate 2; 49, 57 and the upper thread in 60, plate 3).

The large oval, round or sickle shaped bodies found growing on threads, as in fields 50, 51, 58, 66 and 67, plate 3, seem in some cases to have a very soft cell wall as a number of them appear to have been distorted on the microscopic slide. In fields 50 and 51 these bodies



can be seen in all stages of growth. The formation of one of these bodies in a thicker thread is shown in field 52; the cell in field 55b shows an equatorial growth much like it. Field 53 illustrates how scattered these centers of more vigorous growth are.

The lower cell in field 56b, plate 3, might be mistaken for a coccoidal body germinating, but the rod attached to it is the remnant of the mother cell which it would have lost when a little older, just as the older cell above it has lost the mother cell that was attached at the point that still protrudes from this otherwise spherical cell. The upper cell was near the stage when it would entirely disintegrate. In field 57, plate 3, the small rods with oval or spherical swellings look much like spore-bearing rods. These enlargements originated from the growth of a nucleus within the mother cell and the subsequent separation of a portion of this cell, as a small rod, by sagittal segmentation, as may be seen in process just a little above the middle of the field. Three free rods in the field have one pole either pointed or of indefinite outline, a characteristic of sagittal segmentation.

Field 59, plate 3, is interesting as showing the separation of a coccoidal body at one pole of a thread and a rod at the other. The long thread in field 60, plate 3, has a well defined coccoidal body at one pole and a slight swelling at the other as if a nucleus was also in process of growth at that point. The round cell in field 62a has two short processes extending toward the lower part of the picture. This was the only example found in the salt agar cultures that looked like germination. It may be that in some cases a nucleus after forming a coccoidal body, as this one has, is stimulated to produce a secondary growth from daughter nuclei but such instances must be very rare for in the examination of some thousands of slides, only one other was found, fields 11, 14 and 15, plate 1, on which were organisms that had this same appearance. In the latter case a number of such cells were present, while in the former only the one was found on the slide. The appearance of the cell in field 11 might also be due to the growth of a nucleus at the pole of the rod.

The cell in field 65, plate 3, is interesting because it illustrates well the growth of the nucleus in a bud to a large spherical cell. At the left of the organism is a short process at the pole of which is a small bud; at the right is a larger bud at the pole of a much longer process, while above this is a large coccoidal body at the pole of another branch.

*Branched Types:* The term branched types is used to designate those bacterial cells that have a rod-like or thread-like outgrowth at an angle to the major axis of the parent cell.

Branched types exhibit considerable variation. Fields 65, plate 3; 69, 70, 72 and 76 inclusive, and 81 to 83, 85 and 86a and 86b, plate 4, show the differences in this kind of bacterial growth. Fields 72 and 73 were both found on the same slide, the former showing the beginning of a growth such as may be seen in the latter in more mature form. No indications were found in this culture that the branches separate from the parent thread as one is doing by sagittal segmentation in field 75. These branches seem to originate from the growth of a nucleus of a more resistant type in consequence of which they grow in the rod-like form despite the high solution pressure.

The appearance of the organisms in fields 81 to 83 and 85 to 86, plate 4, bear out the indications of a nuclear origin of this form of growth. Sometimes at the pole of the branch a daughter nucleus may grow and produce a coccoidal cell, fields 65, plate 3, and 79, plate 4; or rods, fields 86a and b. The nucleus at the end of the short process in field 34, plate 2, by growing to a coccoidal body or rod would present an appearance like the fields referred to. Fields 81 to 83, plate 4, may be further examples of this secondary growth in which the daughter cell is separating from the branch by sagittal segmentation. The forms in fields 10 and 13, plate 1; 81 to 84 and 86a and b, plate 4, will have to be studied in the hanging drop as there is a possibility that they may be types of conjunction.

*Segmentation:* Segmentation is the name given to the process of bacterial division when the cell wall becomes so softened that the daughter cells separate with either a pointed pole, field 80, plate 4 (to the right of the middle of the picture) or an ill-defined one, field 57, plate 3 (upper left corner).

In field 16 to 21 inclusive, plate 1, are shown examples of cell division on salt agar. Fields 16, 17 and 18 are the types of sagittal segmentation ordinarily seen. The cells in the remaining 3 pictures have the sharper lines of division characteristic of multiplication by fission. The coccoidal cell in the lower right hand corner of field 21 is dividing across the middle, while in the upper left hand corner the coccoidal body is beginning to separate from the parent rod. This group of pictures shows that although much changed morphologically, the fission habit of the species persists. It should be noted that the 2 kinds of division may be found in the same culture for fields 18 to 21 inclusive, were all taken from the same slide.

*Threads:* In addition to the morphologic types that have been described, many of the strains produce long threads. Pictures of these may be found in the various fields. Examples of some of the very long ones are shown in fields 71 and 78, plate 4. Such organisms have

given no indications of gonidium production, but they do divide into rods and threads; the thread in field 71 has divided near the middle into two parts.

In addition to the morphologic types shown under the group headings, some cultures produced on salt agar large cells with flagella-like processes similar to those which Hort<sup>13</sup> found in cultures in 4% glucose broth; field 54, plate 3, contains a picture of such a cell.

In the preliminary work with various mediums one culture was found which produced on starch agar, rod-like organisms at right angles to the mother cell. Field 12, plate A, is the example of this type of growth. Just below the middle of the picture in this field is a double cross; the two growing at right angles to the primary cells cross them at the points of juncture of the three. In this case the secondary growths could originate from polar buds.

Fields 6, 7a and 7b, plate 1, are pictures of the same culture grown under different conditions. It was isolated from the soil by Löhnis and identified as *Bact. pneumoniae*. Its pathogenicity has not been tested. The pictures are shown merely to illustrate the wide variation in morphology of a particular strain at different temperatures and on different mediums.

#### PHYSIOLOGIC REACTIONS

The effect of salt on the physiologic reactions of the bacteria was tested by planting organisms that had grown on salted beef agar in mediums with and without 5% salt and incubating them with similar mediums inoculated with organisms grown on standard beef agar.

The first test was made with cultures that had been transferred on two succeeding days to standard beef agar and then to agar containing approximately 2.5% agar and 6% salt. After one day's growth at 35 C. these cultures were inoculated into standard glucose broth and similar broth containing 5% NaCl, into brom-cresol purple milk and similar milk containing  $\frac{1}{2}$ % NaCl and also into Clark and Lubs' synthetic phthalate medium, both plain and with the addition of 5% NaCl. After one day's growth at 35 C. it was found that among the strains from standard beef agar 8 had produced more gas, 7 the same amount and 7 less gas than the strains from the salted medium. When this test was made the total number of cultures had been reduced by an accident to 22.

The inoculums from standard beef agar produced in one day only from one quarter to one tenth as much gas in the salted glucose broth as in the standard one. The inoculums from salted beef agar showed a good growth in the salted broth, but only five produced gas. In each case only a small bubble was formed.

After 2 days' incubation the only tubes showing any marked difference in gas content were those containing salted glucose broth and inoculated from salted beef agar. These tubes contained between 10-20% gas. Even after 4 days they were, in general, slightly lower in gas content than the check tubes. All except 2 cultures, 17 and 26, rendered the brom-cresol-purple milk with and without salt acid in one day. These 2 cultures were the only ones that failed in 3 days to coagulate the standard milk whether the inoculum was taken from either standard or salted agar. In some instances it was necessary to employ heat to bring about coagulation. Only 3 cultures from standard beef agar coagulated the milk containing 0.5% salt in 4 days; two of these cultures also coagulated it when the inoculum was taken from salted beef agar. After 8 days' incubation the same 11 cultures from both inoculums coagulated the salted milk. The application of heat brought about the coagulation of the others.

Two sets of Clark and Lubs' synthetic mediums were tested, one of the regular formula inoculated with cultures from standard beef agar and the other containing 5% salt inoculated with cultures from salted beef agar. The salted medium showed little or no growth until the third day at 35 C. All tubes were tested on the fifth day for the Voges-Proskauer and the methyl red reactions. In the test for the Voges-Proskauer reaction only 2 cultures, 22 and 23, gave a positive result in the plain broth, and even these were negative in the salted one. Half of the cultures produced the same  $P_H$  concentration in broth medium and half produced a  $P_H$  from 0.3 to 0.7 less in the salted one.

#### DISCUSSION

It has long been recognized that in order to obtain duplicate or nearly duplicate results in tests with bacteria it is necessary to use mediums as nearly the same as our chemical methods will permit. This recognition of the influence of environment on the physiologic activity of bacteria is in accord with the general knowledge that all life responds to its environment. Some forms of life respond to change, both morphologically and physiologically, more than others, and not only different strains of the same species but often individuals of the same strain show marked variation. Since these morphologic changes are known to exist among other living things, it is not surprising that there is some morphologic response among the bacteria. As far as was observed with *B. coli*, however, it is only under extreme conditions of environment, such as on mediums containing 6% salt or, according to other investigators, 4% glucose and 0.2% malachite green, that there is a morphologic response which is sufficiently great to be easily recognized.

The extreme morphologic changes are produced by a selective process, the higher salt concentration tending to kill the weaker forms. Even the organisms that grew on the salted agar the first time showed weakened vitality with succeeding transfers. In order to maintain life, in addition to the usual method of multiplication by fission, they liberate gonidia at the poles or the sides of the rods or threads. The present work has shown definitely that chromatin material is concentrated within the rod into a nucleus and that this nucleus after growing slightly may, as a gonidium, separate from the parent cell and grow independently. This, however, is the exceptional case; the nuclei usually grow while still connected with the mother cell. On standard medium they remain within the mother cell, or there may be a few cells in each culture which produce the nuclei and liberate them as gonidia from the pole of the rod or more rarely from the side of the cell.

On a medium containing 6 or 7% salt and about 2.5% agar the protoplasm seems to be greatly stimulated. The nuclei also respond to this stimulus sometimes by a growth through the side of the mother cell. In some cases in which the sheath of the cell is weak the nucleus in growing retains a spherical shape as a protection against the osmotic pressure. The nucleus in response to the stimulus may start growth anywhere in the axis of the mother cell and produce a spherical body. The coccoidal forms which have only been found on mediums of high osmotic pressure grow until they are spent and begin to disintegrate when they may be found on the microscopic slide as shadow forms. A few that are more resistant retain the power, even in the coccoidal form, to multiply by fission; in some instances division takes place in the diameter of the sphere at right angles to the axis of the parent cell, in others the division takes place at the circumference of the coccoidal body. This evidence shows that the normal processes of the organisms are not destroyed; certain of them only are accelerated, i. e., growth and gonidia production.

The gonidia in growing form either rods or coccoids. The basis for this difference is not known, but it is presumed that those growing in the rod form are more resistant. It may be that the permeability of the cell sheath is a determining factor, for, like Wilson,<sup>7</sup> the author found that coccoidal forms were more plentiful in a medium inoculated from an old culture. In this case the gonidia would get very little protection from the selective permeability of the cell wall.

Some cultures on the salt agar produce nearly all large rods, others short threads and still others exceedingly long threads. A microscopic field generally shows a majority of one kind with some of the others

present. The difference in kinds of morphology produced by the organisms on the salted beef agar may depend on the fundamental quality that has given so much trouble in all bacteriologic work, namely, the inherent differences that exist in strains of the same species, due to their weakness or strength in a particular environment.

The gonidia are the units of reproduction and so apparently carry the inherited characteristics of the strain. The rod-like forms may divide or give off buds but, as they get old, the protoplasm to perpetuate the species forms nuclei which in the old cell of *B. coli* on standard mediums are referred to as polar granules. In a fresh medium these granules swell and meet in the middle of the old sheath. Whether or not they will grow without a sheath is a question which is unsettled for the proof to be gained by cutting the cell wall and observing the response of the nucleus thus exposed has not yet been attempted.

Organisms from a salted medium grew as fast as those from standard beef agar in milk with or without salt and in standard glucose broth with or without salt, but in the salted broth the organisms from standard agar showed retarded power to form gas while those from salted agar formed no gas in one day. From the fact that there appeared to be little or no growth in the Clark and Lubs' salted medium for the first two days, while the broths were cloudy and the milk acid, it seems that the odd morphologic forms and the gonidia recover their normal vitality quicker in the presence of complex organic buffers, like peptone or casein, than in the presence of inorganic ones. This is true even when the solution pressure is high enough to retard free growth.

The discovery of the importance of gonidia in propagating the species under certain conditions introduces a new factor that must be considered in any question dealing with bacterial multiplication. This is especially true in problems of pathology in which the gonidia as filtrable viruses may invade a host that the parent cell could not enter.

The results of other investigators of *B. coli* as here reviewed agree with those of the present report. It is accordingly evident that when placed under a variety of conditions different strains of this species have given an index of the extent of their life history and very likely that of some other non-spore-formers as well. It was found that the normal rod may be stimulated to produce threads or coccoidal forms. Gonidia may be liberated from these cells and become large organisms. If the nucleus grows in the parent cell, it may produce a branch or a globular body. The branch, like the parent, may produce gonidia or rods. On standard mediums the few gonidia produced grow to normal rods; the other morphologic types are rare.

## SUMMARY

This investigation was made with strains of *B. coli*, *B. aerogenes* and four unidentified cultures. The conclusions drawn are considered as applying only to *B. coli*, although they may be equally true of many non-spore-forming species.

The cultures employed, in general, were very stable, but as a rule one or two different strains would show some variation in morphology on all the special mediums, like egg starch agar, etc.

When grown on standard beef agar, *B. coli* multiply almost entirely by fission but some produce gonidia. The number of the latter is usually small, varying with the strain. One culture was found which produced gonidia in abundance.

The gonidia when liberated on standard mediums grow to rods.

When transplanted to a medium of high osmotic pressure many rods die. The resultant growth is due either to the mass action of the organisms or to the presence of more resistant cells in the culture.

The vitality of a culture is reduced by repeatedly transferring to mediums of high solution pressure. All degrees of sensitiveness to this environment have been found.

Some rods and threads under special stimulus produce coccoidal bodies which arise from the growth of a nucleus within the parent cell. This type of growth was obtained on a medium of high solution pressure, i. e., 6% salt and 2.5% agar in the standard beef agar.

These coccoidal growths may separate from the parent cell by the division characteristic of cells with either firm or soft walls. The division is accordingly sharply defined, as typically occurs in fission, or it may be drawn out as in the case of sagittal segmentation. Both kinds of division are found in the same culture.

The coccoidal cells may liberate small cells by sagittal segmentation.

The large free coccoidal bodies become shadow forms and disintegrate if left on the medium which produced them.

A rod-like growth may originate within a mother cell and extend through the side wall of the parent.

Odd shaped cells are usually found in cultures grown on mediums of high solution pressure.

Those cultures that readily respond morphologically to a change in environment show a tendency on rich nitrogenous mediums, like egg agar or egg starch agar, to form small coccoidal bodies at 37 C. and rods, larger than those on standard mediums, at 17 C. The great majority of the organisms tested in this work showed only slight variation under these conditions.

The different morphologic types quickly revert to the normal laboratory type of *B. coli* when planted on a standard medium from one that has caused variation.

In mediums of high solution pressure all *B. coli* retained their physiologic activity though one or more functions of the strains were much suppressed. Gas formation was greatly retarded in all cultures and growth was delayed in Clark and Lubs' synthetic medium; the latter effect was very likely due to the absence of an organic buffer in this solution.

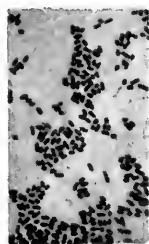
#### EXPLANATION OF PLATE 1

Plate 1.—Magnification in all fields  $\times 1000$ . All films stained with cold aqueous fuchsin.

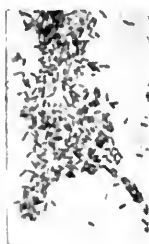
- 1 Culture 2, beef agar, 2 days at 28 C.
- 2 Culture 13, beef agar, 2 days at 28 C.
- 3 Culture 11a, beef agar, 2 days at 28 C.
- 4 Culture 10, beef agar, 2 days at 28 C.
- 5 Culture 4, beef agar, 1 day at 37 C.
- 6 *Bact. pneumoniae*, beef agar, 1 day at 37 C.
- 7a *Bact. pneumoniae*, egg agar, 1 day at 19 C.
- 7b *Bact. pneumoniae*, starch agar, 1 day at 19 C.
- 8a Culture 14, egg starch agar, 1 day at 17 C.
- 8b Culture 14, egg starch agar, 1 day at 37 C.
- 9a Culture 27, egg starch agar, 17 day at 37 C.
- 9b Culture 27, egg starch agar, 1 day at 17 C.
- 10 Culture 10, beef agar, 1 day at 28 C.
- 11 Culture 16, ammonium lactate broth, 1 day at 28 C.
- 12 Culture 24, starch agar, 1 day at 37 C.
- 13 Culture 10, ammonium citrate broth, 5 days at 28 C.
- 14 Culture 16, ammonium lactate broth, 1 day at 28 C.
- 15 Culture 16, ammonium lactate broth, 1 day at 28 C.
- 16 Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.
- 17 Culture 18, beef agar plus 6% NaCl, 1 day at 37 C.
- 18 Culture 26, beef agar plus 6% NaCl, 1 day at 37 C.
- 19 Culture 26, beef agar plus 6% NaCl, 1 day at 37 C.
- 20 Culture 26, beef agar plus 6% NaCl, 1 day at 37 C.
- 21 Culture 26, beef agar plus 6% NaCl, 1 day at 37 C.



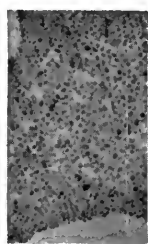
PLATE 1



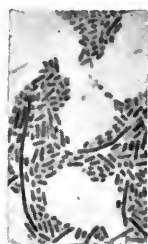
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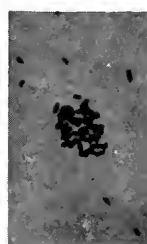
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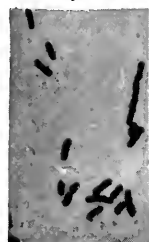
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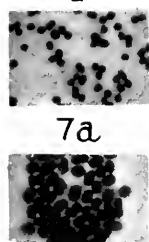
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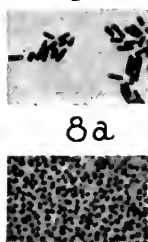
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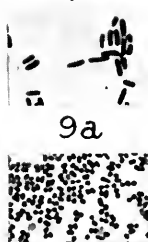
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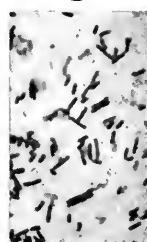
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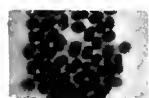
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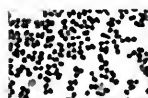
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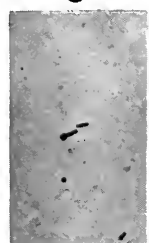
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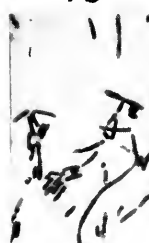
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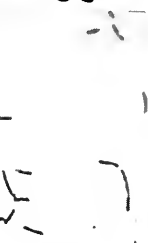
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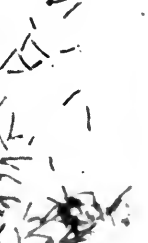
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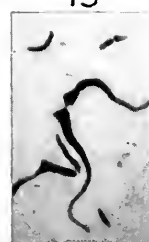
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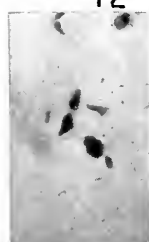
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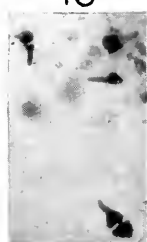
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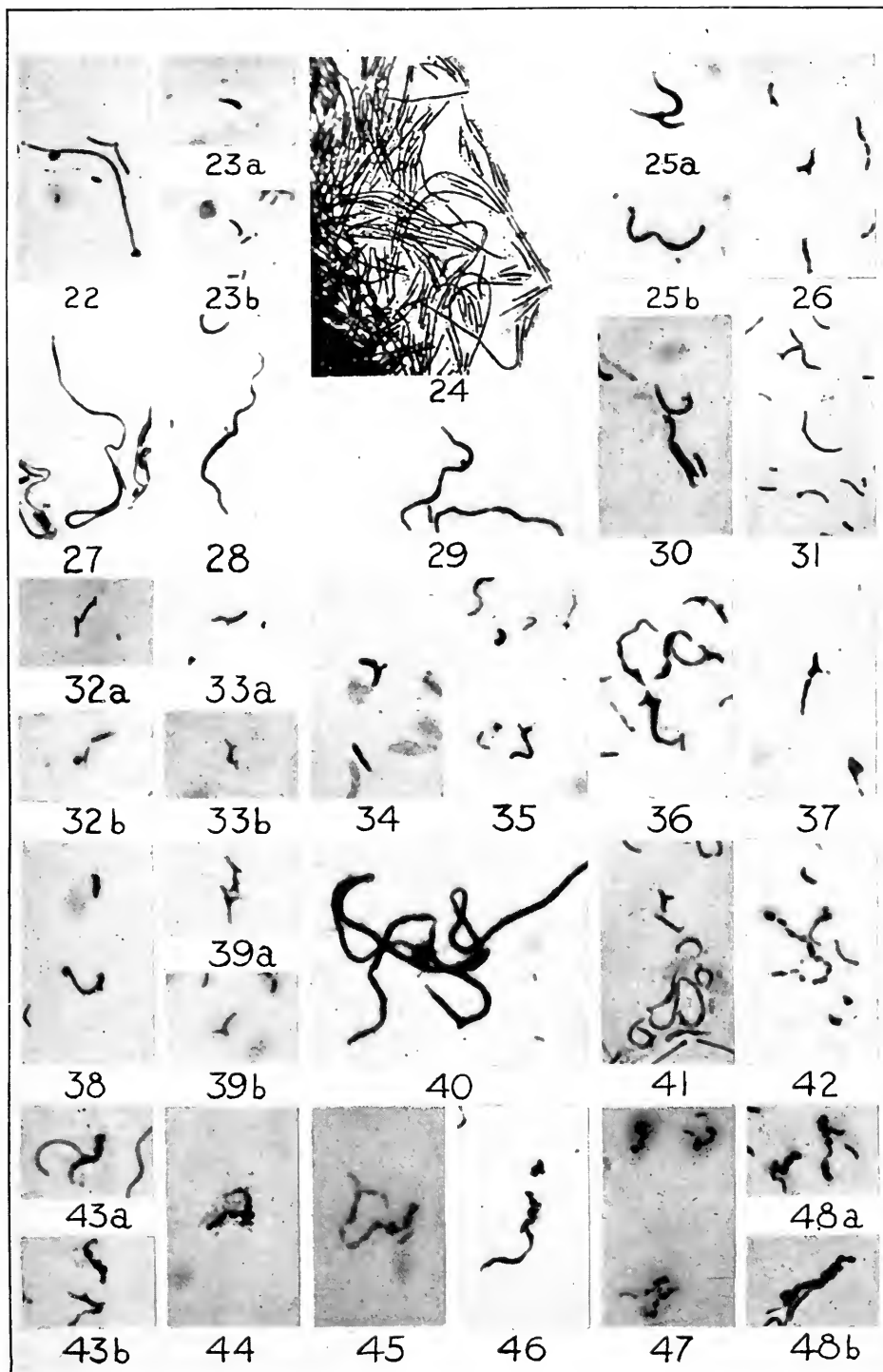
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## EXPLANATION OF PLATE 2

Plate 2.—Magnification in all fields  $\times 1000$ . All films stained with cold aqueous fuchsin.

- 22 Culture 11a, beef agar plus 6% NaCl, 1 day at 35 C.
- 23a Culture 2, beef agar plus 7% NaCl, 1 day at 35 C.
- 23b Culture 6, beef agar plus 7% NaCl, 7 days at 35 C.
- 24 Culture 10, ammonium lactate agar, 1 day at 37 C.
- 25a Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.
- 25b Culture 18, beef agar plus 6% NaCl, 1 day at 35 C.
- 26 Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.
- 27 Culture 11a, beef agar plus 6% NaCl, 1 day at 35 C.
- 28 Culture 18, beef agar plus 6% NaCl, 1 day at 35 C.
- 29 Culture 18, beef agar plus 6% NaCl, 1 day at 35 C.
- 30 Culture 11, beef agar plus 6% NaCl, 3 days at 35 C.
- 31 Culture 14, beef agar plus 6% NaCl, 1 day at 35 C.
- 32a Culture 14, beef agar plus 6% NaCl, 1 day at 35 C.
- 32b Culture 6, beef agar plus 8% NaCl, 1 day at 35 C.
- 33a Culture 10, beef agar plus 6% NaCl, 1 day at 35 C.
- 33b Culture 25, beef agar plus 6% NaCl, 1 day at 35 C.
- 34 Culture 20, beef agar plus 6% NaCl, 1 day at 35 C.
- 35 Culture 1, beef agar plus 6% NaCl, 1 day at 35 C.
- 36 Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.
- 37 Culture 26, beef agar plus 6% NaCl, 1 day at 35 C.
- 38 Culture 1, beef agar plus 6% NaCl, 1 day at 35 C.
- 39a Culture 19, beef agar plus 7% NaCl, 3 days at 35 C.
- 39b Culture 25, beef agar plus 6% NaCl, 1 day at 35 C.
- 40 Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.
- 41 Culture 11, beef agar plus 6% NaCl, 3 days at 35 C.
- 42 Culture 25, beef agar plus 1% NaCl, 1 day at 35 C.
- 43a Culture 6, beef agar plus 7% NaCl, 1 day at 35 C.  
Inoculated from day old growth on 6% NaCl.
- 43b Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.
- 44 Culture 11, beef agar plus 6% NaCl, 18 hours at 35 C.  
Inoculum 15 days old.
- 45 Culture 6, beef agar plus 7% NaCl, 1 day at 35 C.  
Inoculated from 1 day growth on 6% NaCl agar.
- 46 Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.
- 48a Culture 16, beef agar plus 6% NaCl, 1 day at 35 C.
- 48b Culture 11a, beef agar plus 6% NaCl, 1 day at 35 C.

PLATE 2



### EXPLANATION OF PLATE 3

Plate 3.—Magnification in all fields  $\times 1000$ . All films stained with cold aqueous fuchsin.

49 Culture 1, beef agar plus 6% NaCl, 1 day at 35 C.

50 Culture 4, beef agar plus 7% NaCl, 1 day at 35 C.

Inoculated from 1 day growth on 6% NaCl agar.

51 Culture 10, beef agar plus 6% NaCl, 18 hours at 35 C.

Inoculated from 15 day growth on plain agar.

52 Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.

53 Culture 1, beef agar plus 7% NaCl, 1 day at 35 C.

54 Culture 1, beef agar plus 7% NaCl, 1 day at 35 C.

55a Culture 4, beef agar plus 7% NaCl, 1 day at 35 C.

Inoculated from 1 day growth on 6% NaCl agar.

55b Culture 2, beef agar plus 6% NaCl, 1 day at 35 C.

56a Culture 4, beef agar plus 7% NaCl, 1 day at 35 C.

Inoculated from 1 day growth on 6% NaCl agar.

56b Culture 4, beef agar plus 7% NaCl, 7 days at 35 C.

57 Culture 20, beef agar plus 6% NaCl, 1 day at 35 C.

58 Culture 4, beef agar plus 7% NaCl, 1 day at 35 C.

Inoculated from 6% NaCl agar.

59 Culture 14, beef agar plus 6% NaCl, 1 day at 35 C.

60 Culture 19, beef agar plus 6% NaCl, 3 days at 35 C.

61 Culture 6, beef agar plus 8% NaCl, 1 day at 35 C.

62a Culture 25, beef agar plus 6% NaCl, 1 day at 35 C.

62b Culture 3, beef agar plus 6% NaCl, 2 days at 35 C.

63a Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.

63b Culture 4, beef agar plus 7% NaCl, 7 days at 35 C.

64 Culture 2, beef agar plus 7% NaCl, 1 day at 35 C.

65 Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.

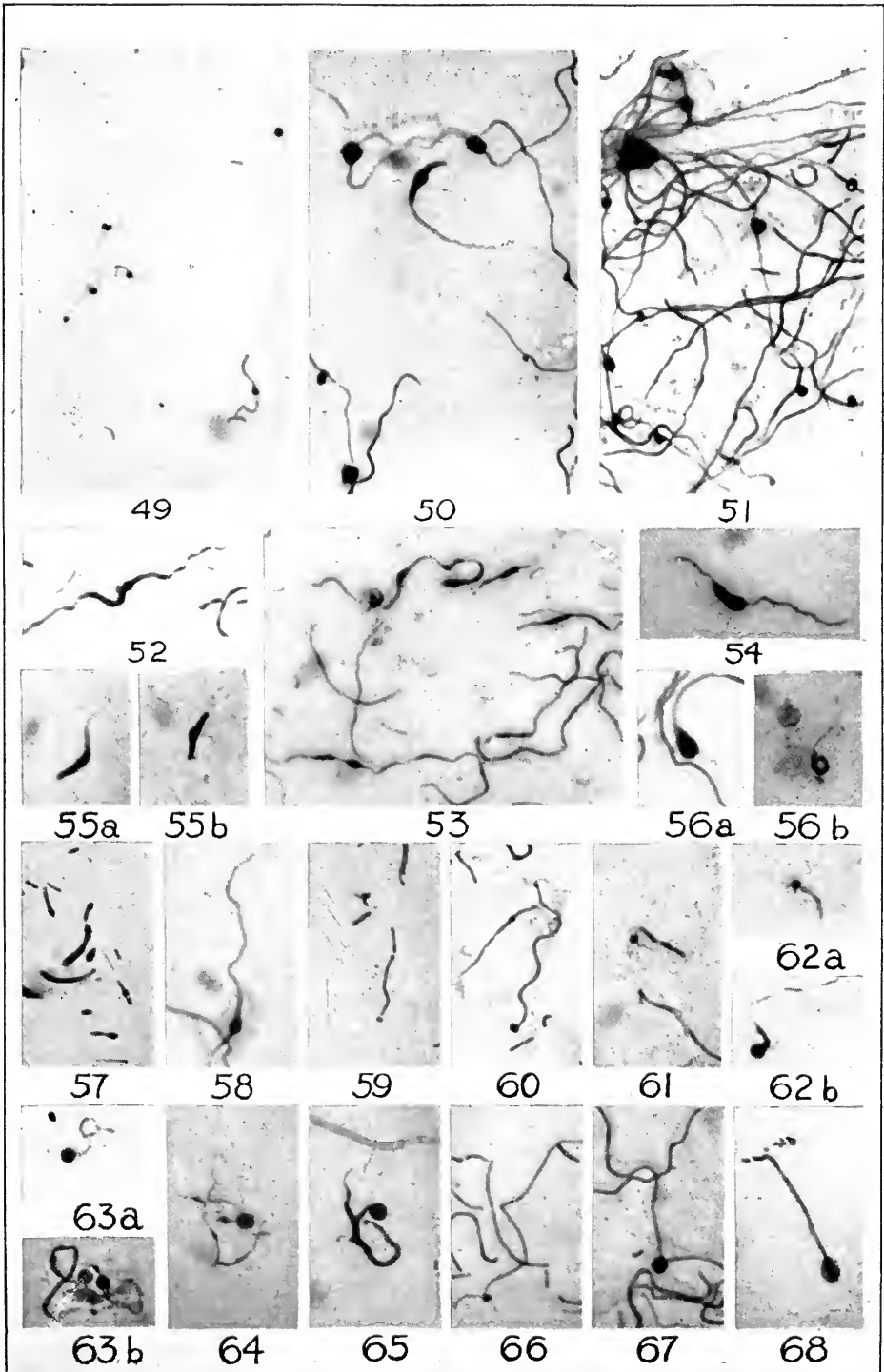
66 Culture 14, beef agar plus 6% NaCl, 1 day at 35 C.

67 Culture 4, beef agar plus 7% NaCl, 1 day at 35 C.

Inoculated from 1 day growth on 6% NaCl agar.

68 Culture 3, beef agar plus 7% NaCl, 1 day at 35 C.

PLATE 3



#### EXPLANATION OF PLATE 4

Plate 4.—Magnification in all fields  $\times 1000$ . All films stained with cold aqueous fuchsin.

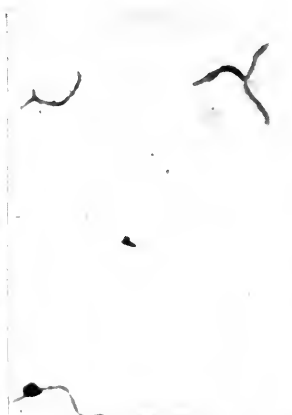
- 69 Culture 3, beef agar plus 7% NaCl, 1 day at 35 C.
- 70 Culture 10, beef agar plus 6% NaCl, 1 day at 35 C.
- 71 Culture 1, beef agar plus 7% NaCl, 1 day at 35 C.
- 72 Culture 4, beef agar plus 7% NaCl, 7 days at 35 C.
- 73 Culture 4, beef agar plus 7% NaCl, 7 days at 35 C.
- 74 Culture 10, beef agar plus 6% NaCl, 18 hours at 35 C.

Inoculum 15 days on standard beef agar.

- 75 Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.
- 76 Culture 11a, beef agar plus 6% NaCl, 3 days at 35 C.
- 77 Culture 27, beef agar plus 6% NaCl, 1 day at 35 C.
- 78 Culture 1, beef agar plus 7% NaCl, 1 day at 35 C.
- 79 Culture 3, beef agar plus 8% NaCl, 2 days at 35 C.
- 80 Culture 20, beef agar plus 6% NaCl, 1 day at 35 C.
- 81 Culture 26, beef agar plus 6% NaCl, 1 day at 35 C.
- 82 Culture 2, beef agar plus 7% NaCl, 1 day at 35 C.
- 83 Culture 11, beef agar plus 7% NaCl, 3 days at 35 C.
- 84 Culture 18, beef agar plus 7% NaCl, 1 day at 35 C.
- 85 Culture 2, beef agar plus 7% NaCl, 1 day at 35 C.
- 86a Culture 11, beef agar plus 6% NaCl, 3 days at 35 C.
- 86b Culture 6, beef agar plus 7% NaCl, 1 day at 35 C.



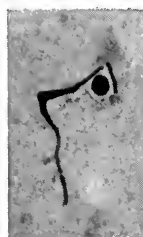
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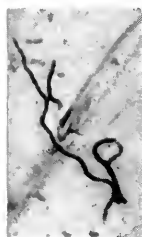
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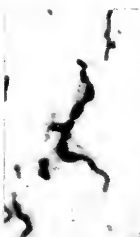
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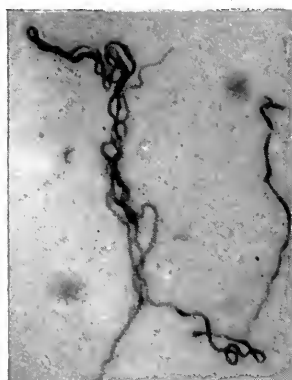
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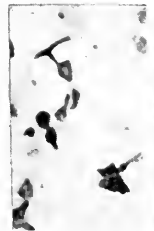
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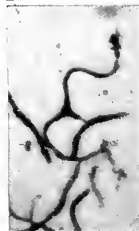
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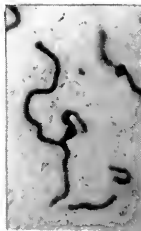
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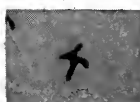
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85



86a



86b





# QUANTITATIVE RELATIONS BETWEEN AMBOCEPTOR AND THE SERUM OF COMPLEMENT-DEFICIENT GUINEA-PIGS

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Through the courtesy of Dr. F. A. Rich, head of the veterinary department of the Vermont Agricultural Experiment Station, I received a number of guinea-pigs from a breed naturally deficient in complement. According to Moore,<sup>1</sup> this characteristic property is inheritable. Both Moore and Downing (cited by Moore<sup>1</sup>) found that in some instances as much as 1 c c of the serum produced no hemolysis of sensitized corpuscles. Moore also found that the apparent lack of complement in the blood serum of some guinea-pigs is not due to the presence therein of anything interfering with the action of the amboceptor. Coca<sup>2</sup> recently reported that both the mid-piece and the end-piece of the complements of these guinea-pigs are present. "There is lacking only the so-called 'third piece,' addition of inactive guinea-pig or human serum containing this third component to the deficient serum causes hemolysis in about three times the minimal hemolytic quantity of normal guinea-pig's serum." The third component according to this author is not identical with the lipid cytozyme (thrombokinas), since the blood of the complement-deficient guinea-pigs clots normally.

Having found 2 guinea-pigs, the serums of which failed to hemolyze 0.5 c c of a 5 % suspension of sensitized sheep corpuscles in doses of 0.8 and 1.0 c c, it was thought of interest to find out whether an increase of amboceptor alone will produce activation of the deficient serum. The amboceptor was employed in doses of 2, 10, 50, 100 and 500 units. The unit of cells was 0.5 c c of a 5% suspension of sheep corpuscles. The complement-deficient serums were employed in doses of 0.1 c c. The incubation period was 1 hour at 37 C. Table 1 shows clearly the effect of the increased amboceptor content. Complete hemolysis occurred in the tube containing 500 units of amboceptor. The complement was employed in doses of 0.1 c c, thus 8 and 10 times

Received for publication, Aug. 1, 1921.

<sup>1</sup> J. Immunol., 1919, 4, p. 425.

<sup>2</sup> Proc. Soc. Exper. Biol. & Med., 1920, 18, p. 71.

less than an amount which in itself failed to hemolyze 0.5 c.c. of a 5% suspension of corpuscles sensitized with 2 units of amboceptor. The serums of both guinea-pigs reacted equally, while in none of the control tubes was hemolysis observed.

In a subsequent experiment an attempt was made to activate and accelerate the reaction of the deficient serum by the addition of inactive normal guinea-pig, human, dog, horse, sheep and rabbit serums. As a control egg albumin was used. Jonas<sup>3</sup> was able to activate by means of inactivated serums complement which had been inactivated by

TABLE 1  
THE EFFECT OF INCREASED AMBOCEPTOR UNITS ON THE ACTIVATION OF THE SERUM OF  
COMPLEMENT-DEFICIENT GUINEA-PIGS

Guinea-Pigs	Amount of Serum in C c	Units of Amboceptor per 0.5 C c	Cell Suspension, 5 % in C c	Result	Remarks
1	1.0	2	0.5	No lysis	
2	0.5	2	0.5	No lysis	
		500	0.5	No lysis	Agglutination
		Salt sol.	0.5	No lysis	
		Salt sol.	0.5	No lysis	To this tube 0.1 c.c. glycerol was added*
1	0.1	...	0.5	No lysis	
2	0.1	...	0.5	No lysis	
1	0.1	2	0.5	No lysis	
	0.1	10	0.5	Trace of lysis	
	0.1	50	0.5	Slight lysis	Agglutination
	0.1	100	0.5	Marked lysis	Agglutination
	0.1	500	0.5	Complete lysis	Agglutination
2	0.1	2	0.5	No lysis	
	0.1	10	0.5	Trace of lysis	
	0.1	50	0.5	Marked lysis	Agglutination
	0.1	100	0.5	Almost complete lysis	Agglutination
	0.1	500	0.5	Complete lysis	Agglutination

\* Amboceptor was glycerolated 50%.

cobra venom; the power of these various serums varied in their capacity for activation. He found horse serum to be a weak activator, while swine serum proved to be extraordinarily strong, acting in dilutions from 1:20,000 to 1:40,000 of 1 c.c. Of 15 human serums only 4 activated the cobra venom inactive complement. In our experiment with complement-deficient guinea-pig serums and the sheep hemolytic system, both human and guinea-pig serums acted powerfully. The human serum employed, however, was pooled. Horse serum was a weak accelerator and sheep and rabbit serums still weaker or they did

<sup>3</sup> Ztschr. f. Immunitätsf., 1913, 17, p. 539.

not act at all. Dog serum acted less vigorously than guinea-pig serum. Egg albumin failed entirely, indicating that serum alone contributes some substance or condition which accelerates the reaction. As controls the various inactive serums were added to cells with increasing doses of amboceptor. It was found that the heated guinea-pig serum (0.1 c c) added to 500 units of amboceptor and 0.5 c c of 5% corpuscles led to complete hemolysis of the agglutinated cells. Dog serum gave marked hemolysis while the other serums remained inactive. In the presence of inactive human serum, complete lysis took place with 0.1 c c of guinea-pig serum, while 0.005 c c of this serum acted only in the presence of 500 units of amboceptor. The smallest quantity of activator (human serum) necessary for complete lysis was 0.0001 c c in the presence of 500 units of amboceptor; 0.001 c c of human serum increased distinctly the effect of deficient guinea-pig serum.

#### DISCUSSION

These observations indicate that a greatly increased amboceptor content is capable of rendering the sheep cells so sensitive that the complement-deficient serum induces lysis in doses which in themselves are entirely inactive in the presence of the usual amboceptor amounts. Whether the amboceptor itself supplements the so-called third component of Ritz<sup>4</sup> is difficult to state. The addition of normal inactive rabbit serum to this system as an activator practically failed, while the addition of normal inactive human or guinea-pig serums greatly enhanced the reaction.

Of particular interest is the fact that in the presence of such large amounts of amboceptor following agglutination some normal inactive guinea-pig serum may produce lysis. There seems to be a similarity in the behavior of the deficient serums to cobra venoms inactive serums or serums whose complement has been inactivated by yeast cells and bacteria as indicated by Coca. When mixed with small quantities of normal inactive serums the complement-deficient serum produces lysis in smaller doses and also with increased velocity. The question whether this is due to an unknown third component or a certain physical state is still problematic. The results indicate clearly that different inactive serums have a variable degree of activating power, thus in large part confirming the work of Jonas.

<sup>4</sup> Ztschr. f. Immunitätsf., 1912, 13, p. 62.

## SUMMARY

The serums of complement deficient guinea-pigs have been studied and their great deficiency as noted by Downing and Moore confirmed.

The deficient action is not due to amboceptor interference because the increase of amboceptor leads to hemolysis of the cells. These serums when employed in comparatively small doses (0.1 cc) and in the presence of 500 units of hemolysin readily cause lysis.

The deficient serums react in a similar manner as cobra venom inactive serums in that the addition of normal inactive homologous or heterologous serums will markedly enhance hemolysis, confirming the results of Coca.

Various inactive serums have varying degrees of activating power when added to the deficient serum amboceptor cell mixture. The same phenomenon was observed by Jonas in the case of cobra venom inactivated serum.

By the increase of amboceptor and the addition of normal inactive homologous or heterologous serums the deficient serum has been made to act within the usual lytic range of normal guinea-pig serum.

## SOME CHARACTERISTICS OF *B. CHAUVÆI*

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### INTRODUCTION

This paper deals with the methods of isolation of *B. chauvœi* from infected tissue and with its differentiation from the other anaerobes frequently found in blackleg and blackleg-like infections.

The organisms with which *B. chauvœi* is most frequently confused is *Vibrio septique*. The latter, as well as *B. chauvœi*, may at times be responsible for acute infections associated with emphysematous lesions, caused by gas-producing anaerobes in cattle. Both *Vibrio septique* and *B. chauvœi* are nonproteolyzers. This facilitates their differentiation from most other anaerobes in such infections.

A review of the literature on blackleg is superfluous as Heller<sup>1</sup> recently has covered the subject. A more concise summary of methods of differentiating anaerobes is to be found in the British Medical Research Committee Bulletins 12 and 39.

### PREPARATION OF MEDIUMS

The mediums used in our study are the Hibler medium; 2% dextrose agar; liver broth; and liver agar.

*Hibler Medium*.—Beef liver, 500 gm., and brain, 500 gm., are ground; 1,000 cc of water added to the liver. Both are cooked in flowing steam one hour. The liver broth is strained through cheese cloth and cotton, 1% peptone and 0.5% salt added. The broth titrated to  $P_H$  8.2. The medium is tubed; two parts of broth used to one part of brain. The tubes are autoclaved under 15 pounds' pressure for one hour.

*Dextrose Agar*.—One % peptone, 0.5% sodium chloride and 2% dextrose are added to beef infusion (beef, 500 gm., to water, 1,000 cc), titrated to  $P_H$  8.2, 2% agar added and heated in flowing steam until the agar is completely dissolved. The medium is clarified with egg albumin, filtered, tubed and autoclaved under 15 pounds' pressure for 20 minutes.

*Liver Broth*.—Liver broth prepared like that used in Hibler medium is clarified with egg albumin, filtered, tubed and autoclaved under 15 pounds' pressure for 20 minutes.

*Liver Agar*.—Two % agar is added to liver broth, heated in flowing steam until the agar is completely dissolved (45 minutes), clarified with egg albumin, filtered, tubed and autoclaved under 15 pounds' pressure for 20 minutes.

Received for publication July 23, 1921.

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<sup>1</sup> Jour. Infect. Dis., 1920, 27, p. 385.

MORPHOLOGY OF *B. CHAUVŒI*

Fifteen strains of *B. chauvœi* were studied, 14 isolated from field cases of blackleg in Kansas, Michigan and Iowa, and one isolated from infected muscle received from the Bureau of Animal Industry in Washington. The morphology and cultural characteristics of these strains are as follows: The organisms occur singly or in pairs, very seldom in chains. They vary in size, but average 0.5 microns in width by 3 to 4 microns in length. They frequently contain granules, and become pleomorphic after incubation for from 24 to 48 hours. The most common variation in form is the clostridial or navicular. The organisms are sluggishly motile in young cultures but actively so in the exudate at the site of inoculation, in guinea-pigs dying from this infection.

Spores develop in from 18 to 24 hours in Hibler medium. They are elliptical, larger than the diameter of the bacterial cell, occupying a median or terminal position in the cell, giving it a lemon-like or pear-shaped appearance. Clostridial forms are found also in the fluid at the site of injection, after death. The morphology of *B. chauvœi* in the body of the guinea-pig differs from that of *Vibrion septique*. The latter frequently grows in long chains on the surface of the liver. This is not true of the former, as it grows singly or in pairs in this location. We have found *B. chauvœi* to be gram-positive in young cultures when stained by Stirling's modification of Gram stain. This is contrary to the assertions of Heller<sup>1</sup> and others. This variation may be due to the fact that these workers did not use the Stirling method. Old cultures become gram-negative.

## CULTURAL CHARACTERISTICS

*Dextrose Agar*.—Agar with 2% dextrose is useful in the differentiation of *B. chauvœi* from other anaerobes, and especially from *Vibrion septique*. The majority of the organisms found in emphysematous lesions grow very well in this medium, when planted in serial dilution in deep tubes. This is not true of *B. chauvœi* which fails to develop at all unless tissue or blood is carried over in the seeding. Even then the colonies formed are small and difficult to isolate. This fact is of great value in establishing the purity of *B. chauvœi* cultures.

*Liver Medium*.—When an extract in physiologic salt solution is made of fresh, sterile guinea-pig liver, and 2 c.c. are added to a tube containing 25 c.c. glucose agar, *B. chauvœi* colonies develop, while failing to do so in glucose agar without extract of liver. In the deep

tubes of beef liver agar described, a good growth is obtained. The colonies develop in from 18 to 24 hours. They are spherical or elliptical in shape, small and translucent. *Vibrio septique*, on the other hand, forms a fluffy colony in this medium.

When the agar concentration is lowered to 1%, *B. chauvœi* grows in colonies very similar in appearance to those of *Vibrio septique* in the 2% agar medium. *B. chauvœi* produces little or no gas in deep tubes of beef liver agar, after incubation for 24 hours. While cultures of *Vibrio septique* show early and extensive gas production, the agar being shattered in 24 hours.

When dilutions of a suspension of *Vibrio septique* are made in beef liver agar and poured into plates, which are then incubated in vacuo, good-sized colonies develop in 24 hours. *B. chauvœi*, on the other hand, fails to show growth, even after incubation for 3 days. This is a differential characteristic of great importance.

*B. chauvœi* ferments liver broth in fermentation tubes, with the formation of both acid and gas. Involution forms appear early, and spores develop rapidly in this medium. *B. chauvœi* will grow without the intervention of anaerobic conditions when placed in liver broth to which cubes of cooked liver have been added.

The most distinctive growth characteristic of this organism in liquid medium is its tendency to agglutinate. The medium remains cloudy for a short time during active growth, but clumping takes place rapidly, leaving the supernatant liquid layer as clear as if no growth had occurred.

*Hibler Medium*.—*B. chauvœi* grows under apparent aerobic conditions in this medium. Gas is produced; rapid agglutination occurs; the cultures have no odor. The brain tissue turns slightly pink, but is not digested. Neither *B. chauvœi* nor *Vibrio septique* give any evidence of proteoclastic activity in these mediums. The only odor is a faint indication of butyric acid. This is most easily detected when old cultures are placed on slides and treated slightly. *Vibrio septique* tends to grow in chains in Hibler medium. This is not true of *B. chauvœi*.

*Litmus Milk*.—*Vibrio septique* grows well when seeded in large amounts, by pipet, into litmus milk which has been freshly boiled to expel oxygen. An acid reaction occurs in 24 hours. No growth is obtained when inoculation is made by loop. In the case of *B. chauvœi* no growth results, even when 5 times the amount used for successful seeding of *Vibrio septique* is employed.

*Gelatin*.—In gelatin prepared with liver broth as a base, *B. chauvœi* forms fluffy colonies. The medium is liquified and gas produced by some strains of *B. chauvœi*, the colonies rapidly settling to the bottom of the tube.

## CARBOHYDRATE FERMENTATION

Beef infusion was inoculated with *B. coli*, incubated 24 hours at 37 C. to remove sugar. The colon organisms were removed by coagulation with egg white and 1% peptone and 2% carbohydrate added. The broth was then titrated to  $P_H$  8.2, filled into fermentation tubes, autoclaved 20 minutes at 15 pounds' pressure, tested for sterility and then inoculated with 0.1 cc of a 48-hour Hibler culture. Readings were taken after incubation at 37 C. for 24 and 48 hours. Three lots of mediums were made at different times and 3 tubes inoculated with each organism. Table 1 shows the average results of these inoculations.

TABLE 1  
FERMENTATION TESTS

Carbohydrate	Malignant Edema (Novy)	B. Ghon- Sachs (Meyer)	Vibrio septique (Pasteur)	B. chauvœi (5 Strains Were Tested)	B. welchii 14	B. Botu- linus 71
Glucose.....	A G	A G	A G	No growth	A G	A G
Saccharose.....	A	A	A	No growth	A G	A
Maltose.....	A G	A G	A G	No growth	A G	A G
Lactose.....	A	A G	A G	No growth	A G	A
Xylose.....	A	A	A	No growth	A G	A
Dulcitol.....	A	A	A	No growth	A	A
Sorbitol.....	A G	A	A	No growth	A	A G
Glycerin.....	A	A	A	No growth	No growth	A
Inulin.....	A	A	A	No growth	A G	A
Salicin.....	A	A G	A G	No growth	A	A
Mannite.....	A	A	A	No growth	A G	A
Liver broth.....	A G	A G	A G	A G	A G	A G
Levulose.....	A G	A G	A G	No growth	A G	A
Dextrin.....	A	A	A	No growth	No growth	A
Raffinose.....	A	A	A	No growth	A	A
Amygdalin.....	A	A	A	No growth	No growth	A
Glycogen.....	A	A G	A G	No growth	A G	A

G, gas; A, acid.

ISOLATION OF *B. CHAUVœI* FROM INFECTED MUSCLE

When suspected blackleg tissue is received it is examined microscopically. A small piece of the material is macerated in sterile salt solution, and a few cc of the suspension drawn into a capillary pipet and heated at 60 C. for 45 minutes.

These suspensions, heated and unheated portions, are seeded into liver agar and 2% glucose agar, serial dilutions being made. Hibler medium is also inoculated. Finally, a guinea-pig is injected subcutaneously with 1 cc of each suspension. Residual material is dried under aseptic precautions, for future use. This is done in vacuo at 37 C., or at atmospheric pressure in an oven at 55 C.

After incubation at 37 C. for 18-24 hours, the liver agar tube is examined for characteristic colonies. These are fished and planted



again in liver agar, glucose agar and Hibler medium. Great care must be exercised in picking the colonies, especially if anaerobes other than *B. chauvœi* are present.

These alternations should be repeated until purity is assured by morphologic study, failure to grow in 2% glucose agar, animal inoculations and protection tests. It is reiterated here that failure to grow in serial dilutions of deep tubes of glucose agar, along with characteristic growth of the same material in liver-agar, is a most important test for purity of the culture.



Fig. 1.—*B. chauvœi* from 16 hour Hibler culture, X 1,200; Gram stain, Stirling.

If the inoculated guinea-pig dies it should be examined promptly, gross lesions noted, smears made from the site of injection, and liver surface; and cultures made from the heart blood in liver agar, glucose agar and Hibler medium.

If the guinea-pig dies in less than 16 hours, death is probably due to a more invasive organism than *B. chauvœi*. For this reason *B. chauvœi* may easily be lost when it, in combination with *Vibrio septique*, is injected into a guinea-pig and cultures are made from the heart blood. For the purpose of separating blackleg from this organism guinea-pigs passively immunized with specific antitoxic serum may be used. By protecting the guinea-pigs against *Vibrio septique* and not *B. chauvœi*, death usually results from blackleg and the organism may be isolated in this way.

PATHOGENICITY OF *B. CHAUVOEI*

*Guinea-Pigs.*—The guinea-pig is the animal of choice in the experimental study of *B. chauvoei*. The cultures used in this investigation seem to vary considerably in virulence for these animals. At the same time, certain variations occurring in successive cultural and animal passages of one and the same strain, seriously weaken statements in regard to the variations in virulence between different strains.

Thus, strain 1, 24-hour Hibler culture, after passage through a guinea-pig, killed a 350 gm. animal in a dose of 0.02 c.c. The same



Fig. 2.—Impression from liver surface of guinea-pig injected with pure culture of *B. chauvoei*; Gram stain, Stirling; X 1,200.

strain, 24-hour Hibler culture, after this passage, required 0.2 c.c to kill another guinea-pig of the same weight. These strange variations might be referred to differences in resistance of individual animals, uncontrollable variation in the same or different lots of culture medium. Or, again, organisms of markedly different virulence might occur side by side in a given strain, the more virulent type predominating at one time, the less virulent at another.

Vibrien septique produces more rapidly fatal infections in guinea-pigs than does *B. chauvoei* following the injection of like amounts of culture. The infections by the latter are characterized by a marked

swelling around the injection site some hours before death. When *B. chauvæi* infections are rapidly fatal, the blood at death may be devoid of organisms. If such animals are allowed to remain at room temperature for from one to two hours after death, the causative organisms appear on the liver surface and in the heart blood. On the other hand, *Vibrio septique* is of a more invasive character, organisms invariably being found in the heart blood at death.

The guinea-pigs in table 2 presented typical blackleg lesions. Those receiving 0.05 c.c. of culture did not die until the third day following injection. The hair over the lesions on these animals was easily removed. This is usually the case when the animals die slowly, and may occur in animals dying rapidly, due to the local tissue changes incident to the rapid bacterial multiplication beneath the skin.

TABLE 2

RELATIVE SUSCEPTIBILITY OF GUINEA-PIGS, WHITE MICE AND PIGEONS TO *B. CHAUVÆI*

Animal	Weight in Gm.	Amount Injected in c.c.	Result		
			24 Hours	48 Hours	72 Hours
Guinea-pig 1.....	350	0.1	Dead		
2.....		0.1	Dead		
3.....		0.05	Slight swelling	Marked swelling	
4.....		0.05	Swelling	Marked swelling	Dead
5.....		0.025	Swelling	Dead	
White mouse 1.....	20	0.5	Dead		
2.....		0.5	Dead		
3.....		0.2	.....	Survived	
Pigeon 1.....	350	0.5	.....	Survived	

All injections made subcutaneously with *B. chauvæi* strain No. 3.

The entire abdominal and thoracic regions were hemorrhagic, certain areas of muscle being more deeply stained than others, especially in the axillary regions. There was a small amount of hemorrhagic fluid present and slight serogelatinous infiltration of the intramuscular tissue. A few small gas bubbles were present in the axillary spaces. No appreciable amount of gas was present in the intestines. The entire subcutaneous lesion brightened in color on exposure to air.

Bacteria at site of injection: Gram-positive bacilli in large numbers; many clostridia.

Bacteria on liver surface: Gram-positive bacilli appearing singly or in pairs, seldom in chains or filaments.

Pure culture of *B. chauvæi* was obtained from the heart blood.

White mice, according to von Hibler,<sup>2</sup> are refractory to *B. chauvæi* infection. We have found these animals to be susceptible to cultures highly virulent for guinea-pigs. However, mice are considerably more resistant, 2 or 3 times the dose fatal for guinea-pigs being required to produce infection in the first-mentioned animals.

After death there was slight subcutaneous edema at the point of inoculation.

Bacteria at site of inoculation: Gram-positive bacilli and clostridia.

Bacteria on liver surface: Gram-positive bacilli, singly and in pairs.

<sup>2</sup> Kolle de Wassermann, Handb. d. path. Mikroorg., 1912, 4, p. 788.

Pigeons, which are well known to be highly susceptible to *B. welchii*, are strongly refractory to *B. chauvœi* inoculations, according to von Hibler,<sup>2</sup> Arloing, Cornevin and Thomas.<sup>3</sup> The relative susceptibility of these animals is illustrated in table 2. Although the pigeon in table 2 did not die, we have been successful in producing fatal blackleg infections in pigeons. The animals in table 2 were injected simultaneously with the same culture.

The pectoral region was deeply hemorrhagic, friable and moist. The tissues were separated by tiny gas bubbles. The lesions resembled blackleg lesions in cattle having the sweet sickish odor characteristic of blackleg lesions in cattle.

Bacteria at site of inoculation: Gram-positive rods; clostridia.

Bacteria on liver surface: Gram-positive rods single and in pairs. Pure culture of *B. chauvœi* from heart blood.

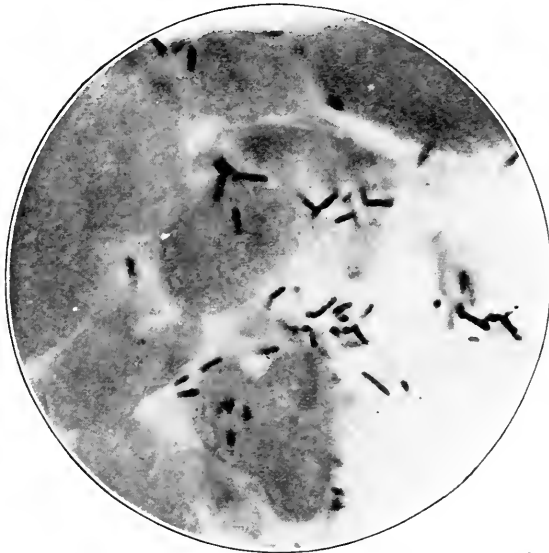


Fig. 3.—Section of calf muscle showing *B. chauvœi*; Gram stain, X 1,200.

Frequent mention is made in the literature regarding blackleg in sheep. Von Hibler<sup>2</sup> is of this opinion. Kitt<sup>4</sup> states, "In sheep whose caudal skin is not so close to the vertebra as in cattle, but has a substratum of spongy cell tissue, the inoculation into the tail apex produces a marked swelling and general infection; by application of an ice-bag, however, the local reaction can be prevented."

On the other hand, it is an interesting fact that we have never isolated true *B. chauvœi* from the muscle of sheep alleged to have

<sup>2</sup> Le charbon Symptomatique du boef, 1887, p. 87.

<sup>4</sup> Kolle u. Wassermann, Handbuch. d. path. Mikroorg., 1912, 4, p. 821.

died from a field case of blackleg. Anaerobes pathogenic for guinea-pigs were isolated, but invariably they were varieties other than the typical *B. chauvœi*. It was, therefore, decided to attempt the experimental infection of sheep with true *B. chauvœi* cultures. For this purpose cultures highly virulent for guinea-pigs were used.

Table 3 shows the results of intramuscular inoculation of *B. chauvœi* 15 into a lamb, a kid and a sheep. A Hibler culture grown 24 hours at 37 C. and placed in the refrigerator for two weeks was used. The lamb and sheep developed slight swellings at the point of injection and were lame. The kid succumbed to the infection.

TABLE 3  
INOCULATION OF SHEEP, LAMB AND KID

Animal	Culture	Age of Culture, Weeks	Dose in c c	Results			
				18 Hours	24 Hours	28 Hours	96 Hours
Guinea-pig.....	15	2	0.1	Sick	Sick	Dead	
Kid.....	15	2	2.0	Leg swollen	Sick	Dead	
Lamb.....	15	2	2.0	Leg swollen	Sick	Lame	Recovered
Sheep.....	15	2	2.0	Leg swollen	Sick	Lame	Recovered

TABLE 4  
INOCULATION OF SHEEP

Animal	Culture	Age of Culture	Dose in c c	Results		
				18 Hours	24 Hours	48 Hours
Guinea-pig.....	09	1 month	0.05	Sick	Sick	Dead
Sheep.....	09	1 month	5.0	Sick	Dead	
Guinea-pig.....	11	2 days	0.05	Sick	Sick	Dead
Sheep.....	11	2 days	5.0	Sick	Sick	Dead
Guinea-pig.....	13	5 months	0.02	Sick	Dead	
Sheep.....	13	5 months	0.50	Sick	Dead	
Guinea-pig.....	15	1 month	0.02	Sick	Dead	
Sheep.....	15	1 month	5.0	Sick	Dead	
Guinea-pig.....	16	2 months	0.02	Sick	Dead	
Sheep.....	16	2 months	5.0	Sick	Dead	
Guinea-pig.....	M	1 month	0.1	Dead		
Sheep.....	M	1 month	5.0	Dead		

Inoculations were made in the hind leg.

The results given in table 4 show that young, healthy sheep can be infected with *B. chauvœi* by injecting large doses of virulent culture.

One sheep in table 4 was injected with a 24-hour Hibler culture of an organism (M) sent to us as a culture of *B. chauvœi* isolated from a sheep supposedly dead of blackleg. The organism did not resemble our strains of *B. chauvœi* culturally. It did, however, resemble *Vibrio septique* culturally and in its pathogenicity for small animals.

*Kid*.—Serohemorrhagic edema; no gas in tissues; small amount of gas in the intestines; gram-positive bacilli single and in pairs on surface of the liver; pure culture of *B. chauvœi* from the heart blood.

*Sheep*.—All animals showed lesions similar to those found in cattle dying from blackleg infection; bloody exudate from nostrils and swelling of affected leg; muscle dark reddish black with characteristic blackleg odor; considerable congestion throughout abdominal cavity; liver light yellow in color; spleens enlarged and softened. Pure cultures of *B. chauvœi* were obtained from the heart blood of all the sheep injected with strains of *B. chauvœi*.

The sheep injected with culture M. died 5 hours earlier than the sheep injected with *B. chauvœi*. The clinical symptoms and lesions were similar to those of blackleg, but the organism isolated from the heart blood was a *Vibrio septique* group organism which grew luxuriantly in glucose agar.

It would appear from tables 3 and 4 that while sheep are somewhat refractory to injections of *B. chauvœi*, fatal infections may be produced with large doses of highly virulent cultures. Field cases of blackleg in sheep are rarely found in comparison to the frequency with which it occurs in cattle. This undoubtedly is due to the relative insusceptibility of sheep to natural infection with *B. chauvœi*.

In common with previous observers, it has been found that rabbits are highly refractory to large doses of virulent *B. chauvœi*. White rats are also resistant to such cultures.

#### IMMUNOLOGIC TESTS

*Protective Power of Antiblackleg Serum (Horse) Against B. Chauvœi and Vibrio Septique*.—While data of value in differentiating *B. chauvœi* from *Vibrio septique* have been obtained, as presented in the foregoing pages, it was considered desirable to discover a specific immunity reaction as an ultimate means of differentiation. Roux and Chamberland<sup>5</sup> state that animals vaccinated against blackleg resist *Vibrio septique* infections. Their opinion is shared by Duenschmann<sup>6</sup> who claims that antiblackleg serum protects against *Vibrio septique* infections. On the other hand, Leclainche and Vallée,<sup>7</sup> supported by immunity experiments, insist that there is a clear-cut difference between these organisms. The results about to be presented bear out the latter opinion.

Guinea-pigs were injected subcutaneously with 0.1 c.c. of antiblackleg serum. This serum was obtained from horses hyperimmunized with virulent cultures of *B. chauvœi*. Twenty-four hours after the injection of this serum, 2 of the guinea-pigs were injected with large

<sup>5</sup> Ann. de l'Inst. Pasteur, 1887, 1, p. 561.

<sup>6</sup> Ibid., 1894, 8, p. 402.

<sup>7</sup> Ibid., 1900, 14, p. 590.

amounts of virulent culture of *B. chauvæi* 8. Two others were injected with a virulent culture of *Vibrion septique* (Pasteur). Controls, injected with normal horse serum, received varying doses of the same cultures. The inoculations were made subcutaneously with 24-hour Hibler cultures. The results of this experiment are striking and are summarized in table 5.

Animals 1-4 and 12-14 showed typical blackleg lesions.

Reddish fluid oozed through the skin of animals 8-11. The subcutaneous and intramuscular tissues were slightly hemorrhagic, containing a sero-hemorrhagic fluid and small gas bubbles. The tissues were soft and jelly-like in appearance. The intestines were covered with small hemorrhages, considerable gas being present. The heart blood was partially coagulated.

Site of Injection: Gram-positive bacilli, chains, filaments and clostridia.

Liver Surface: Gram-positive bacilli, chains and filaments.

TABLE 5

PROTECTIVE POWER OF ANTIBLACKLEG SERUM AGAINST *B. CHAUVÆI*; FAILURE TO PROTECT AGAINST *VIBRION SEPTIQUE*

Guinea-Pig	Serum		<i>B. chauvæi</i> 8	<i>Vibrion septique</i> (Pasteur)	Result		
	Anti-Black-leg	Normal Horse Serum			24 Hours	48 Hours	96 Hours
1	—	—	0.02	—	Slight swelling	Dead	Survived Survived Survived
2	—	—	0.05	—	Swelling	Dead	
3	—	—	0.1	—	Dead		
4	—	—	0.1	—	Dead		
5	0.1	—	0.2	—	.....	.....	
6	0.1	—	0.5	—	.....	.....	
7	—	—	—	0.05	.....	.....	
8	—	—	—	0.1	Sick	Dead	
9	—	—	—	0.2	Dead		
10	0.1	—	—	0.1	Dead		
11	0.1	—	—	0.2	Dead		
12	..	0.1	0.1	—	Dead		
13	..	0.1	0.2	..	Dead		
14	..	0.1	0.5	..	Dead		

Table 5 shows that 0.1 c.c. antiblackleg serum protects against 25 lethal doses of *B. chauvæi*. On the other hand, a like amount fails entirely to protect against even one fatal dose of *Vibrion septique*. This method is of great advantage in determining the purity of questionable cultures, and in isolating the *Vibrion septique* from *B. chauvæi*, both in cultures and in infected muscle, in case of coexistence of these two species in the same tube or the same muscle, as shown in table 6.

Guinea-pigs weighing 350 gm. were injected with antiblackleg serum, anti-*Vibrion septique* serum (Pasteur), normal horse serum and a mixture of antiblackleg and anti-*Vibrion septique* serums. The guinea-pigs were inoculated subcutaneously 24 hours later with 0.2 c.c. of

20-hour Hibler cultures of *B. chauvœi* (7) and *Vibrio septique* (Pasteur). The results show that antiblackleg serum and anti-*Vibrio septique* serum give specific protection against *B. chauvœi* and *Vibrio septique* respectively. The guinea-pigs receiving normal horse serum died from a mixed infection while those receiving both antiblackleg and anti-*Vibrio septique* serums survived.

Guinea-pigs 1, 2, 3 and 4 presented typical pictures of *Vibrio septique*, chains and filaments on the liver. Pure cultures of *Vibrio septique* were obtained from the heart blood in each case.

Guinea-pigs 5 and 6 presented blackleg pictures—no chains or filaments on the surface of the liver. Pure cultures of *B. chauvœi* were obtained from the heart blood in each case.

The lesions in guinea-pigs 7 and 8 resembled blackleg lesions, but long chains and filaments were found on the surface of the liver. Liver agar cultures from the blood of these animals showed the presence of *B. chauvœi* and *Vibrio septique*.

TABLE 6  
ANTIBLACKLEG AND ANTI-VIBRIO SEPTIQUE SERUMS

Guinea-Pig	Serum			<i>B. chauvœi</i>	<i>Vibrio Septique</i>	Results	
	Anti-blackleg	Anti- <i>Vibrio Septique</i> (Pasteur)	Normal Horse			24 Hours	96 Hours
1	0.1	.....	.....	0.2	0.2	Dead	
2	0.1	.....	.....	0.2	0.2	Dead	
3	0.2	.....	.....	0.2	0.2	Dead	
4	0.2	.....	.....	0.2	0.2	Dead	
5	.....	0.4	.....	0.2	0.2	Dead	
6	.....	0.5	.....	0.2	0.2	Dead	
7	.....	.....	0.3	0.2	0.2	Dead	
8	.....	.....	0.5	0.2	0.2	Dead	
9	0.2	0.2	.....	0.2	0.2	Survived	Survived
10	0.2	0.4	.....	0.2	0.2	Survived	Survived

#### AGGLUTINATION REACTIONS

Statements as to the value of agglutination reactions in the differentiation of anaerobes are conflicting. Many authors find them to indicate close relationship between the various species, and so to be valueless in their separation. Others, particularly MacIntosh and Fildes,<sup>8</sup> state that the agglutination reaction is of great value in separating *B. chauvœi* and *Vibrio septique*.

The first difficulty to be overcome in such a study is the rapid sedimentation of *B. chauvœi* cultures. Believing this flocculability to be caused by electrolytes, such cultures were subjected to repeated washing with distilled water, and were finally suspended in this medium.

<sup>8</sup> Brit. Med. Research Committee Bull. 12.



TABLE 7  
AGGLUTINATION OF *B. CHAUVÆI* AND *VIBRION SEPTIQUE* BY NORMAL HORSE SERUM

Normal Horse Serum, 0.5 C c	Suspension <i>Vibrio septique</i>	Final Dilution	Agglutination
1:10	0.5 c c	1:20	+++
1:20	0.5 c c	1:40	+++
1:40	0.5 c c	1:80	+++
1:80	0.5 c c	1:160	+++
1:100	0.5 c c	1:200	++
1:200	0.5 c c	1:400	++
1:400	0.5 c c	1:800	++
1:800	0.5 c c	1:1600	—
1:1000	0.5 c c	1:2000	—
		Control	—
	Suspension <i>B. chauvæi</i>		
1:10	0.5 c c	1:20	++
1:20	0.5 c c	1:40	++
1:40	0.5 c c	1:80	++
1:80	0.5 c c	1:160	++
1:100	0.5 c c	1:200	++
1:200	0.5 c c	1:400	—
1:400	0.5 c c	1:800	—
1:800	0.5 c c	1:1600	—
1:1000	0.5 c c	1:2000	—
		Control	—

+++ = complete agglutination; ++ = incomplete agglutination; + = no agglutination.

TABLE 8  
AGGLUTINATION OF *B. CHAUVÆI* AND *VIBRION SEPTIQUE* BY ANTIBLACKLEG SERUM

Antiblackleg Serum, 0.5 C c	Suspension <i>B. chauvæi</i>	Final Dilution	Agglutination
1:10	0.5 c c	1:20	+++
1:20	0.5 c c	1:40	+++
1:40	0.5 c c	1:80	+++
1:80	0.5 c c	1:160	+++
1:100	0.5 c c	1:200	+++
1:200	0.5 c c	1:400	+++
1:400	0.5 c c	1:800	+++
1:800	0.5 c c	1:1600	—
1:1000	0.5 c c	1:2000	—
		Control	—
	Suspension <i>Vibrio septique</i>		
1:10	0.5 c c	1:20	++
1:20	0.5 c c	1:40	++
1:40	0.5 c c	1:80	++
1:80	0.5 c c	1:160	++
1:100	0.5 c c	1:200	++
1:200	0.5 c c	1:400	—
1:400	0.5 c c	1:800	—
1:800	0.5 c c	1:1600	—
1:1000	0.5 c c	1:2000	—
		Control	—

Fermentation tubes of liver broth were inoculated with *B. chauvœi* cultures incubated 18 hours at 37 C. and centrifuged. The organisms were suspended in distilled water and washed 5 times, and finally suspended in a volume of distilled water equal to one-half the original culture. Purified cresol, 0.2%, was used as a preservative. This suspension was allowed to stand at room temperature for at least 48 hours. The supernatant only was used when sedimentation took place. Suspensions of *Vibrio septique* were prepared in the same manner. Suspensions of the same opacity were used.

Such suspensions are perfectly stable and serve very well in agglutination tests. Normal horse serum and antibrucella serum were tested against suspensions of *Vibrio septique* and *B. chauvœi*.

It was found that normal horse serum agglutinates both *B. chauvœi* and *Vibrio septique*, the former in titer 1:200, the latter in 1:400. Horse antibrucella serum agglutinates *B. chauvœi* in titer 1:800 and *Vibrio septique* in 1:200. It would appear, therefore, that there is a distinct zone of specificity in these reactions, despite the rather high agglutination titer of the normal horse serum, especially for *Vibrio septique*. To resolve this question it will be necessary to titer the serum of a large number of normal horses against both of these organisms. The titers obtained should be compared with an equally large number of those from brucella immune horses. If a satisfactory zone of specificity is found to exist, these tests should be supplemented by careful absorption reactions. For the rest, it must be admitted that this reaction is distinctly in the experimental stage and requires much further elaboration and study.

#### SUMMARY

The methods of preparation of mediums suitable for the growth of *B. chauvœi* are described.

*B. chauvœi* is discussed with regard to cultural and morphologic characteristics.

A method for the rapid isolation of *B. chauvœi* from infected material is given.

The failure of pure cultures of *B. chauvœi* to grow on 2% dextrose agar is an important and much neglected criterion for judging the purity of such cultures.

Cultures of *B. chauvœi* of high virulence for guinea-pigs are fatal to mice, though in much higher doses than are necessary to kill guinea-pigs.

The amount of *B. chauvœi* culture required to kill a pigeon is many times greater than that required to kill a guinea-pig.

Sheep are apparently somewhat refractory to natural infection with *B. chauvœi* but can be successfully infected with large doses of virulent cultures. No strain of genuine *B. chauvœi* has been isolated from the tissues of sheep suspected of having died of blackleg.

Kids may be fatally infected with pure cultures of *B. chauvœi*.

Protection tests with antibrackleg serum indicate a marked specific for *B. chauvœi*, and assist materially in its identification and its differentiation from *Vibrion septique*.

It is possible that the agglutination reaction may be of use in the identification of this organism, but its study is still distinctly in the experimental stage.

## STUDIES ON COMPLEMENT FIXATION

### II. THE VELOCITY OF FIXATION OF COMPLEMENT IN THE WASSERMANN TEST \*

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The marked difference of opinion among workers with regard to the time and temperature of fixation of complement in the Wassermann test can perhaps best be illustrated by the fact that two recently standardized Wassermann procedures recommend, in one case,<sup>1</sup> a fixation period of 40 minutes in the water bath and, in the other,<sup>2</sup> either 18 hours in the icebox or 3 to 4 hours in the icebox plus 1 hour in the water bath. The methods of fixation employed by different workers known to the writers embrace 30, 40 or 60 minutes in the water bath, 2 hours in the icebox plus 30 minutes in the water bath, and 4, 8, 10, 12 or 18 hours in the icebox. That all these methods give a relatively high degree of correct results is, in our opinion, not due to the fact that they are all correct, but rather to the nature of the Wassermann test. The majority of syphilitic serums possess such marked complement binding power that fixation periods ranging from 5 to 15 minutes are frequently quite ample. Simon<sup>3</sup> has indeed utilized this fact in his Wassermann tests, using for each test 2 fixation periods, 5 minutes and 1 hour, in the water bath. If a given test is positive after 5 minutes, he obviously needs go no further; if negative, the results are based on the hour fixation period. It is with the weakly positive serum that the method of fixation plays an important rôle. A longer fixation period would obviously increase the fixability of such serum. In attempting to prolong their fixation unduly, however, a practical difficulty presents itself. Complement gradually deteriorates at any temperature, and there is danger of confusing specific fixation with complement deterioration.

Received for publication, July 29, 1921.

\* A preliminary report by Kahn, R. L.: Soc. for Exper. Biol. and Med., 1921, 18, p. 168.

<sup>1</sup> Hinton, W. A.: Jour. Syph., 1920, 4, p. 598.

<sup>2</sup> Kolmer, J. A.: Matsunami, T., and Trist, M. E.: Jour. Syph., 1921, 5, p. 63. This paper as well as one by Dean, Jour. Path. & Bacteriol., 1917, 21, p. 193, give an extensive review of the literature.

<sup>3</sup> Jour. Amer. Med. Assn., 1917, 72, p. 1535.

Perhaps the outstanding feature of the generally accepted views of fixation is that short periods (one-half to one hour) at water bath temperature give parallel results with prolonged periods (4 to 18 hours) at icebox temperature. The logic of this assumption is clear. The complement-fixation reaction being biologic in nature, it presumably takes place more rapidly at 37.5 C. than at lower temperatures. That this assumption does not hold true in complement fixation with purified proteins has been shown by one of us in a previous paper.<sup>4</sup> The rate of fixation of complement with immune rabbit serum and protein antigens was found to be similar at water bath, room or icebox temperature, with a slight tendency for stronger fixation at the latter temperature. It was further shown that the maximum degree of fixation was obtained after 4 hours at icebox temperature. To determine whether these results are applicable to complement-fixation tests with syphilitic serum and the usual Wassermann antigens has been the aim of this paper.

#### EXPERIMENTS

The complement-fixation tests were carried out with a sheep cell system, employing 2 units of complement, 2 units of amboceptor and 4 to 5 units of antigen. These ingredients, as well as the sheep cell suspension, were used in 0.1 c.c. quantities. The syphilitic serums used in these experiments were positive Wassermann serums left over from those sent to this laboratory for examination. These serums were used in every case in the following gradations: 0.01, 0.007, 0.004, 0.003, 0.002, 0.001, 0.0005, 0.0003, and 0.0001 c.c.

Six antigens were employed: (1) an alcoholic extract of beef hearts previously freed from ether soluble lipoids; (2) the same antigen, cholesterinized; (3) a crude alcoholic extract of guinea-pig hearts; (4) a Noguchi antigen; (5) a cholesterinized antigen of human hearts; and (6) a crude alcoholic extract of beef hearts.

#### THE PREPARATION OF THE ANTIGENS

1. The alcoholic extract of beef hearts was prepared according to the method described by Neymann and Gager.<sup>5</sup> Briefly, fresh beef hearts were freed from fat, fiber and blood vessels and then ground and dried. The dried material was then extracted 4 or 5 times with ether. These extractions were carried out for several days at a time in the icebox

<sup>4</sup> Kahn, R. L.: *Jour. Exper. Med.*, 1921, 34, p. 217.

<sup>5</sup> *Jour. Immun.*, 1917, 2, p. 573. Compare Ecker, E. E. and Sasani, K.: *Jour. Infect. Dis.*, 1919, 24, p. 174.

and continued until the supernatant ether showed no coloring matter, the ether being discarded in every case. The beef heart was then completely freed from ether by drying, placed in a flask, and absolute alcohol added in such proportions that a layer of fluid about 1 inch high covered the dried material. The alcoholic extract was ready for use after about 2 weeks' extraction.

2. The cholesterinized antigen was the one just described, except that it was half saturated with cholesterol, or, what is equal to the same thing, 0.4 gm. of cholesterol was added to every 100 c.c.

3. The guinea-pig antigen was prepared by extracting guinea-pig hearts, previously cut into small pieces and dried between filter paper, in absolute alcohol. The extraction was carried out for several months in the icebox. This antigen was kindly given us by Mr. John Koopman, serologist of the New York City Health Department.

4. The Noguchi antigen was prepared from beef hearts in the manner described by Noguchi.<sup>6</sup>

5. The cholesterinized antigen of human hearts was prepared similarly to the guinea-pig heart antigen except that it was half saturated with cholesterol.

6. The crude alcoholic extract of beef hearts was also prepared similarly to the guinea-pig heart antigen.

These antigens were titrated for their antigenic, anticomplementary and hemolytic properties in the usual manner. Five times the quantity of these antigens employed in the tests were neither anticomplementary nor hemolytic.

#### THE EFFECT OF TEMPERATURE ON THE VELOCITY OF FIXATION OF COMPLEMENT IN THE WASSERMANN TEST

The fixation periods employed were 0, 5, 15, and 30 minutes and 1, 2, 3, 4, 5, 6 and frequently 7 hours. The temperatures were icebox (8 to 12 C.), room (18 to 23 C.) and water bath (37.5 C.). The tests were carried out in the usual manner, employing the various gradations of serum, 0.1 c.c. of antigen, 0.1 c.c. (2 units) of complement and 0.1 c.c. of salt solution. After a given fixation period, 0.1 c.c. of the standard sheep cell suspension (5%) and 0.1 c.c. hemolysin (2 units) were added and incubated in the water bath at 37.5 C. for about 15 minutes, when the serum and antigen controls would be completely hemolyzed. All readings were made after keeping the tubes in the icebox over night.

<sup>6</sup> Serum Diagnosis of Syphilis, Ed. 2, p. 79.

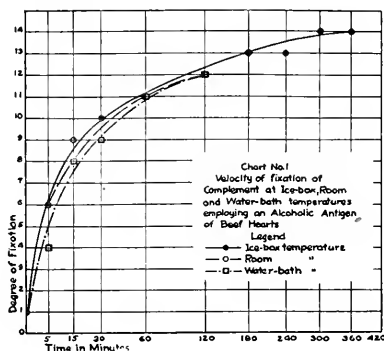
The fixation experiments at water-bath and room temperatures were not extended beyond 2 hours, in view of the marked deterioration of complement after prolonged exposure at these temperatures.

TABLE 1  
VELOCITY OF FIXATION OF COMPLEMENT AT ICEBOX, WATER-BATH, AND ROOM TEMPERATURES  
Tests with Alcoholic Antigen of Beef Heart

Fixation		Wassermann Positive Syphilitic Serum (c c)									Number of Positive Signs Denoting Degree of Fixation
Period	Temperature	0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.0003	0.0001	
0 Min.	Icebox.....	1*	—	—	—	—	—	—	—	—	1
5 Min.	Icebox.....	3	2	1	—	—	—	—	—	—	6
	Water bath.....	2	1	1	—	—	—	—	—	—	4
	Room.....	3	2	1	—	—	—	—	—	—	6
15 Min.	Icebox.....	4	3	1	1	—	—	—	—	—	9
	Water bath.....	3	3	1	1	—	—	—	—	—	8
	Room.....	4	3	1	1	—	—	—	—	—	9
30 Min.	Icebox.....	4	4	1	1	—	—	—	—	—	10
	Water bath.....	4	3	1	1	—	—	—	—	—	9
	Room.....	4	3	1	1	—	—	—	—	—	9
60 Min.	Icebox.....	4	4	2	1	—	—	—	—	—	11
	Water bath.....	4	4	2	1	—	—	—	—	—	11
	Room.....	4	4	2	1	1	—	—	—	—	12
2 Hrs.	Icebox.....	4	4	2	1	1	—	—	—	—	12
	Water bath.....	4	4	2	1	1	—	—	—	—	12
	Room.....	4	4	2	1	1	—	—	—	—	12
3 Hrs.	Icebox.....	1	4	2	2	1	—	—	—	—	13
4 Hrs.	Icebox.....	4	4	2	2	1	—	—	—	—	13
5 Hrs.	Icebox.....	4	4	3	2	1	—	—	—	—	14
6 Hrs.	Icebox.....	4	4	3	2	1	—	—	—	—	14

\* 4 = +++++, 3 = +++, 2 = ++, 1 = +, and — = negative.

Forty-five different syphilitic serums were used in these experiments, and because of the general uniformity of the findings only 2 tables will be given. Table 1 gives the results of one experiment carried out with the alcoholic extract antigen of beef hearts.

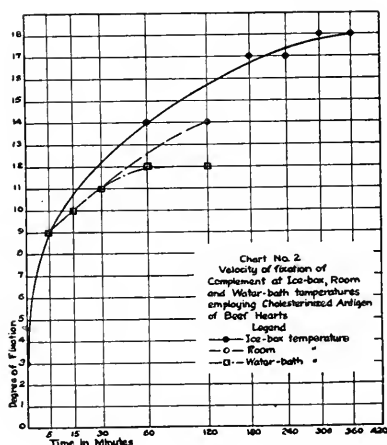


It is evident from table 1 that the velocity of fixation of complement is practically the same at icebox, room, and water bath temperatures. The degree of fixation is reduced to a numerical value by adding the

number of plus signs after each fixation period. Chart 1 is based on the findings recorded in this table. It will be noted that the hour divisions on the time axis (abscissae) are so spaced that each one is equal to the two thirds of the power of the preceding division. In this

TABLE 2  
VELOCITY OF FIXATION OF COMPLEMENT AT ICEBOX, WATER-BATH, AND ROOM TEMPERATURES  
Tests with Cholesterinized Antigen of Beef Heart

Fixation		Wassermann Positive Syphilitic Serum (c c)									Number of Positive Signs Denoting Degree of Fixation
Period	Temperature	0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.0003	0.0001	
0 Min.	.....	2	1	—	—	—	—	—	—	—	3
5 Min.	Icebox.....	4	3	1	1	—	—	—	—	—	9
	Water bath.....	4	3	1	1	—	—	—	—	—	9
	Room.....	4	3	1	1	—	—	—	—	—	9
15 Min.	Icebox.....	4	4	1	1	—	—	—	—	—	10
	Water bath.....	4	4	1	1	—	—	—	—	—	10
	Room.....	4	4	1	1	—	—	—	—	—	10
30 Min.	Icebox.....	4	4	2	1	—	—	—	—	—	11
	Water bath.....	4	4	2	1	—	—	—	—	—	11
	Room.....	4	4	2	1	—	—	—	—	—	11
60 Min.	Icebox.....	4	4	3	2	1	—	—	—	—	14
	Water bath.....	4	4	3	2	1	—	—	—	—	12
	Room.....	4	4	3	2	1	—	—	—	—	12
2 Hrs.	Icebox.....	4	4	3	2	1	—	—	—	—	14
	Water bath.....	4	4	2	1	1	—	—	—	—	12
	Room.....	4	4	3	2	1	—	—	—	—	14
3 Hrs.	Icebox.....	4	4	4	2	2	1	—	—	—	17
4 Hrs.	Icebox.....	4	4	4	2	2	1	—	—	—	17
5 Hrs.	Icebox.....	4	4	4	3	2	1	—	—	—	18
6 Hrs.	Icebox.....	4	4	4	3	2	1	—	—	—	18



way it was possible to plot 5, 15 and 30 minute values on a relatively large scale and at the same time keep the width of the chart within small limits.



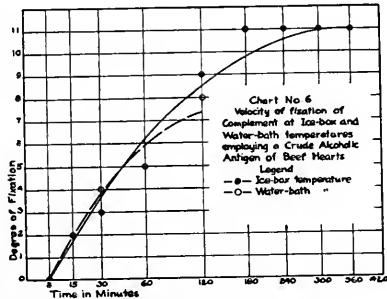
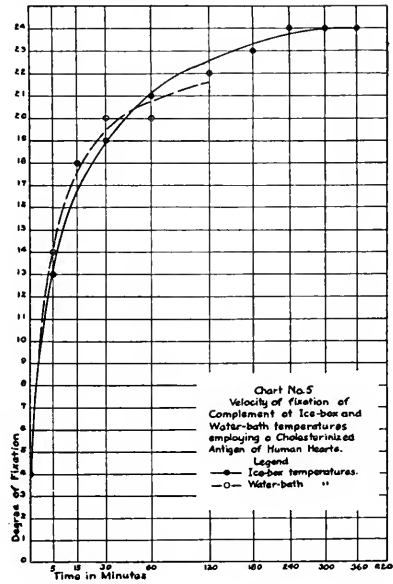
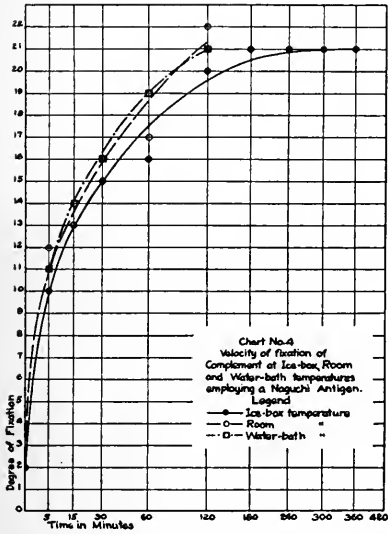
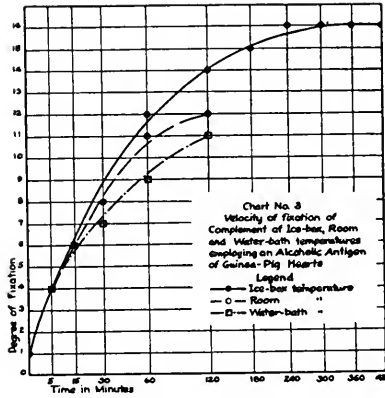
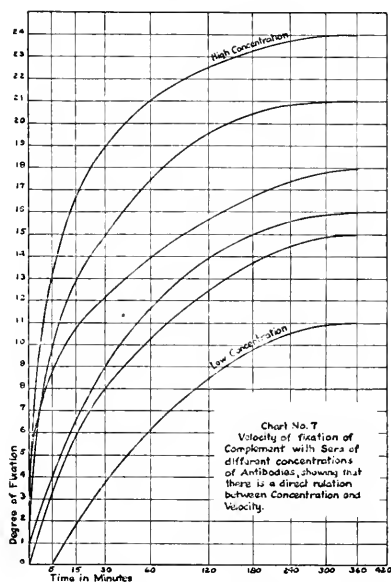


Table 2 and chart 2 give the results of a similar experiment with the cholesterinized antigen of beef heart. The tendency for somewhat stronger fixation at icebox temperature is illustrated by this experiment. Charts 3 to 6, inclusive, represent experiments with different antigens. With the exception of the Noguchi antigen chart, all show the same tendency, namely, either equal fixation at all temperatures or a tendency for stronger fixation at icebox temperature. In the case of the Noguchi antigen (chart 4), however, the tendency for somewhat stronger fixation at room and water bath temperatures than at icebox



temperature is indicated. In several instances icebox temperature gave somewhat stronger fixation with this antigen also. In most cases, the tendency was as indicated in this chart, except that fixation at room temperature was, as a rule, weaker than at water bath temperature.

Another element brought out by these charts is that 4 hours' fixation at icebox temperature approaches the maximum amount of complement that serum and antigen, in the quantities employed in these experiments, are capable of binding. That this does not hold true in every case when employing a Noguchi antigen is indicated in chart 4. Even with this antigen, however, the amount of complement fixed after 4 hours' incubation in the icebox was, in most cases, somewhat greater than after 1 hour in the water bath.

It will be recalled that our complement-fixation studies with protein antigens<sup>4</sup> indicated that the rate of fixation of complement was determined by the concentration of antibodies in the immune serum. That the same holds true with syphilitic serums and Wassermann antigens is indicated in chart 7, which consists of 6 icebox fixation curves representing serums of different concentrations. A study of this chart reveals that a serum of high antibody concentration shows considerable fixation of complement immediately after mixing the ingredients and over 50% of fixation after an incubation period of 5 minutes. With serums of lesser antibody concentration, the curves rise less and less abruptly, and the first signs of fixation do not take place until after from 5 to 15 minutes' incubation.

This chart, as well as the preceding ones, speak against  $\frac{1}{2}$  to 1 hour fixation periods at water bath temperature as well as prolonged fixation periods, such as 12 to 18 hours, at icebox temperature. The employment of a 4-hour fixation period at icebox temperature in the Wassermann test would appear, from our results, to be a dependable procedure.

The well-known tendency of cholesterinized antigens to pick up occasional false positive reactions, however, puts them in a class by themselves. And with the routine Wassermann tests carried out in this laboratory with this antigen, a 4-hour fixation period is, in our opinion, unsafe. We employ this fixation period with our alcoholic antigen tests, but, in the case of the cholesterinized antigen tests, a 1-hour fixation period at icebox temperature is resorted to. In view of the relatively sharper binding power of cholesterinized antigens compared with alcoholic antigens when employed with the same serum, a 4-hour period with the latter antigens gives practically identical results with a 1-hour fixation period with cholesterinized antigens. The main advantage of icebox over water-bath fixation lies in the fact that, at the former temperature complement is practically preserved, while at the latter it deteriorates rapidly.

#### SUMMARY

The velocity of fixation of complement employing syphilitic serums and 6 different Wassermann antigens was studied. The periods of fixation were 0, 5, 15 and 30 minutes and 1, 2, 3, 4, 5, 6 and frequently 7 hours. The temperatures of fixation were water bath, room and icebox, and, in some cases, water bath and icebox.

It was observed that the velocity of fixation of complement is not markedly affected by temperatures ranging between water bath and

icebox. The tendency for slightly stronger fixation at icebox temperature compared with that of the water bath was noted with all antigens, except the Noguchi. The latter antigen showed a tendency for somewhat stronger fixation at water bath temperature.

It was also observed that a fixation period of 4 hours at icebox temperature approaches the maximum amount of fixation of complement with all antigens, including the Noguchi, although the latter in a few cases showed slightly more fixation after 1 hour in the water bath than after 4 hours in the icebox.

Finally, it was shown that the velocity of fixation of complement is directly proportional to the concentration of antibodies in the syphilitic serums.

## STUDIES ON COMPLEMENT FIXATION

### III. THE EFFECT OF HEAT ON COMPLEMENT-FIXING ANTIBODIES

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This paper presents studies on the effect of heat on three types of complement-fixing substances: those present in syphilitic serum; those present in rabbits immunized with purified proteins, and those found in animals as a result of bacterial immunization.

These studies were undertaken as a result of an accidental observation made in this laboratory during the early part of the summer of 1920. A number of Wassermann positive syphilitic serums intended for inactivation were accidentally placed in a water bath of 62 C. (instead of 56 C.) and permitted to remain for 15 minutes. Instead of discarding these serums, they were examined for complement-fixing substances side by side with parts of the same serums which were inactivated for half an hour at 56 C. It was desired to corroborate the prevalent view that a temperature of 62 C. destroys the complement-fixing substance in syphilitic serum.

The Wassermann tests were made in duplicate, with a cholesterinized antigen with a half hour fixation period in the water bath and an alcoholic antigen with a 4 hour fixation period in the icebox. It was found that the tests carried out with water bath fixation were negative, indicating complete destruction of the complement-fixing substances, while those carried out with icebox fixation gave only slightly weaker results than the same serums which underwent a half hour heating at 56 C.

That the difference in the results of the two sets of tests was not due alone to the different antigens employed was soon shown by preliminary experiments. These experiments furthermore established without question that a fixation period of 4 hours in the icebox gave results altogether different from a fixation period of a half hour in the water bath when employing serums exposed to different temperatures. We thus had a problem worthy of experimental investigation. It will be recalled that Noguchi<sup>1</sup> has pointed out the marked destruc-

Received for publication, July 29, 1921.

<sup>1</sup> Serum Diagnosis of Syphilis, Ed. 2, p. 97.

tion of syphilitic antibodies during inactivation; the antibody content he found to be reduced to about one-fourth of the total. He used in his test a half hour fixation period in the water bath, and it seemed likely that a 4 hour period in the icebox would have given him altogether different results. More recently, Kolmer, Rule and Trist,<sup>2</sup> also, studied the effect of heat on complement-fixing antibodies in syphilitic serums. They found that temperatures ranging from 62 to 65 C. destroy these antibodies completely. And with regard to inactivation, they recommend a 15 minute instead of the usual 30 minute period at 56 C. This, because of the high destruction of complement-fixing substances during the latter period of inactivation. They, however, also used water bath fixation.

In view of these considerations, a series of studies on the effect of heat on complement-fixing substances was undertaken with a particular view to determining the effect of the mode of fixation on the destructibility of these antibodies.

#### THE EFFECT OF HEAT ON COMPLEMENT-FIXING SUBSTANCES IN SYPHILITIC SERUMS<sup>3</sup>

The plan of these studies was to find the relative number of complement-fixing substances in unheated serum and in the same serum heated at various temperatures in the water bath for different periods. All tests were carried out in duplication with a 1 hour fixation period in the water bath, 37.5 C., and a 4 hour period in the icebox, 8-12 C.

The complement fixation tests were carried out in the usual manner with a sheep-cell system and guinea-pig complement. The complement, amboceptor, antigen and sheep-cell suspension were used in 0.1 c c quantities while the immune serum was used in every case in the following dilutions: 0.01, 0.007, 0.004, 0.003, 0.002, 0.001, 0.0005, 0.0003, and 0.0001 c c. Two units of complement and 2 units of amboceptor were used, and the tests were uniformly read after permitting the racks to remain in the icebox over night.

The syphilitic serums in these experiments were positive Wassermann serums left over from those sent to this laboratory for examination. The antigens employed were (1) an alcoholic extract of beef heart; (2) the same antigen cholesterinized; (3) an alcoholic extract of guinea-pig hearts; and (4) a Noguichi antigen. These antigens

<sup>2</sup> Jour. of Syph., 1920, 4, p. 641.

<sup>3</sup> For preliminary report see Kahn, R. L., and Boyd, A. G.: Abstr. of Bacteriol., 1921, 5, p. 17.

are the same as the first 4 antigens employed in study II.<sup>4</sup> Five times the quantity of these antigens employed in the tests were neither anticomplementary nor hemolytic.

*Effect of Mode of Fixation on Velocity of Thermal Destruction of Syphilitic Antibodies.*—For the sake of uniformity, the velocity of thermal destruction of syphilitic antibodies was measured by subjecting the serums in practically all cases to the same temperatures and periods. The following were chosen: 5, 15 and 30 minutes at 56 C. and 10 and 20 minutes at 62 C.

At the very beginning of these experiments a phenomenon was encountered which we have not as yet been able to explain fully. We found that some serums appear to possess greater fixing powers after being heated up to 30 minutes at 56 C. than before heating; this occurred only when icebox fixation was resorted to.

An increase in fixability in inactivated serum compared with raw serum is what might be expected in such cases when the latter contains considerable amounts of native complement and hemolysin. The destruction of complement and partial destruction of hemolysin by heat compared with slight destruction of syphilitic antibodies would tend to render the tests stronger after inactivation. The fact, however, that this increase in fixability after heating, takes place only when icebox fixation is employed is difficult to explain.

Table 1 illustrates this point. The antigen was an alcoholic extract of beef hearts and the serum dilutions ranged from 0.01 to 0.0001 c.c. The degree of fixation is reduced to a numerical value by adding the total number of plus signs in each case. The antibody loss or gain due to heating is computed accordingly. Of 32 serums tested, 10 showed gains in antibody content after heating up to 30 minutes at 56 C. These gains ranged from 5 to 125%. The others showed either no antibody loss at this temperature or only about 10% loss; this, only when a fixation period of 4 hours in the icebox was employed. With the employment of water-bath fixation, the average antibody loss due to heating was 32%.

The velocity of thermal destruction of syphilitic complement fixing substances was next studied, employing a cholesterinized antigen of beef hearts. Thirty-four serums were tests and the experiments conducted as outlined in table 1. The results up to 30 minutes heating at 56 C. were similar to the findings with the alcoholic antigen, except that a

<sup>4</sup> Kahn, R. L., and Olin, R. M., Jr.: Jour. Infect. Dis., 1921, 29, p. 630.

comparatively fewer number showed gains in antibody content. Ten serums tested with the alcoholic extract of guinea-pig hearts also gave results similar to the alcoholic extract antigen of beef hearts. Eleven serums tests with the Noguchi antigen showed considerable loss due to heating with either icebox or water-bath fixation, more so, however, with the latter.

TABLE 1

THE EFFECT OF THE MODE OF FIXATION ON THE VELOCITY OF THERMAL DESTRUCTION OF SYPHILITIC ANTIBODIES. TESTS WITH ALCOHOLIC-EXTRACT ANTIGEN OF BEEF-HEART

Time and Temperature of Heating	Mode of Fixation	Serum C c										Total Plus S gns	Effect of Heating	
													Antibody	
		0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.0003	0.0001	Loss %		Gain %	
0	Water bath* Icebox.....	3† 4	3 4	2 2	1 2	1 1	— —	— —	— —	— —	10 13	.. ..	.. ..	
5 min. at 56 C.	Water bath. Icebox.....	3 4	2 4	1 3	1 2	1 2	— 1	— —	— —	— —	8 16	20 ..	.. 23	
15 min. at 56 C.	Water bath. Icebox.....	3 4	2 4	1 3	1 3	1 2	— 1	— —	— —	— —	8 17	20 ..	.. 30	
30 min. at 56 C.	Water bath. Icebox.....	2 4	2 4	1 3	1 3	— 2	— 2	— —	— —	— —	6 18	40 .	.. 38	
10 min. at 62 C.	Water bath. Icebox.....	1 4	1 3	— 3	— 2	— 1	— —	— —	— —	— —	2 13	80 0	.. 0	
20 min. at 62 C.	Water bath. Icebox.....	1 3	1 3	— 2	— 1	— 1	— —	— —	— —	— —	2 10	80 23	.. ..	

\* The period of water bath fixation was 1 hour; of icebox fixation, 4 hours.

† 4 = ++++; 3 = +++; 2 = ++; 1 = +; and — = negative.

A temperature of 62 C. was found to be destructive to the complement-fixing substances in practically all serums tested. When employing alcoholic and cholesterinized antigens, the average antibody destruction after 20 minutes' heating at 62 C. was 40% with the icebox fixation and about 70% with water-bath fixation. With the Noguchi antigen marked destruction was noted at this temperature with either mode of fixation.

Table 2 summarizes the differences in antibody destruction with the 2 modes of fixation after heating for 30 minutes at 56 C. and 20 minutes at 62 C.

One of the first problems that suggested itself as a result of these findings was whether the comparatively small loss in fixability of heated serum after 4 hours' fixation in the icebox compared with 1 hour in the water bath was due primarily to the longer period of fixation or



to the colder temperature of fixation. In order to throw light on this question, a series of 30 experiments (of the total) were carried out employing the following three modes of fixation: 1 hour in the icebox, 1 hour in the water bath, and 4 hours in the icebox. It was observed that the differences in the degree of fixation after 1 hour in the icebox compared with 1 hour in the water bath was not marked. When employing the Noguchi antigen, the degree of fixation was slightly stronger after 1 hour in the water bath than after the same period in the icebox. With the alcoholic and cholesterinized antigens the tendency was for slightly stronger fixation after 1 hour in the icebox.

TABLE 2

SUMMARY OF THE EFFECT OF THE MODE OF FIXATION ON THE DESTRUCTION OF SYPHILITIC ANTIBODIES BY HEAT

No. of Serums Tested	Antigen	Mode of Fixation	Average Loss of Antibodies Due to Heating	
			After 30 Min. at 56 C., %	After 20 Min. at 62 C., %
32	Alcoholic Extract of Beef Hearts	1 hour in water bath..... 4 hours in icebox.....	31 22 (gain)	76 42
34	Cholesterinized antigen of Beef Hearts	1 hour in water bath..... 4 hours in icebox.....	32 3	64 41
10	Alcoholic Extract of Guinea-Pig Hearts	1 hour in water bath..... 4 hours in icebox.....	36 7 (gain)	83 38
11	Noguchi Antigen.....	1 hour in water bath..... 4 hours in icebox.....	29 16	77 65
Totals of 87 serums.....		1 hour in water bath..... 4 hours in icebox.....	32 10 (gain)	75 46

As a whole it would appear that the increased fixability of 4 hours in the icebox compared with 1 hour in the water bath is due largely to the longer period of fixation.

Perhaps the outstanding feature of these experiments is the marked variations in the behavior of each serum toward heat. Two syphilitic serums subjected to the same temperatures and tested with the same antigens and the same modes of fixation, will frequently show 100% variation. The general tendency, however, is as indicated in table 2.

*Summary of Results With Syphilitic Serums.*—The velocity of thermal destruction of syphilitic complement-fixing antibodies was investigated, and it was observed that the mode of fixation markedly affected the results obtained. When fixation was carried out for 1 hour at water bath temperature, the heating of serums for 5, 15 and 30

minutes at 56 C. and 10 and 20 minutes at 62 C. showed progressive destruction of these antibodies, corroborating the findings of Noguchi and other investigators. When, however, fixation was carried out for 4 hours at icebox temperature, the heating of serums up to 30 minutes at 56 C. showed either a small gain or slight loss in antibody content. A temperature of 62 C. resulted in almost half of antibody destruction compared with water-bath fixation. These findings apply to alcoholic and cholesterinized antigens. When employing a Noguchi antigen, antibody destruction due to heat was found to be marked with either mode of fixation, although more so with water-bath than with icebox fixation.

With regard to the inactivation of serums to be tested, for half an hour at 56 C., the results indicate that there is no advantage in reducing this period to 10 or 15 minutes when employing alcoholic and cholesterinized antigens and icebox (4 hours) fixation. With the Noguchi antigen, however, even with icebox fixation, a reduction of the inactivation period should be of advantage.

#### THE EFFECT OF HEAT ON COMPLEMENT-FIXING ANTIBODIES PRODUCED BY PROTEIN IMMUNIZATION <sup>5</sup>

Having shown to what extent the mode of fixation affected the heat destruction of complement-fixing substances of syphilitic serums, it seemed worth while to extend these studies to specific complement-fixing antibodies produced in rabbits by protein immunization.

Four rabbits were employed in this series. Two were immunized with edestin from hempseed and 2 with phaseolin from the kidney bean. The methods of immunization are fully described in the first paper of this series.<sup>6</sup> The complement-fixation tests were conducted in the same manner as with the syphilitic serums. The specific protein antigens were tested with the rabbit immune serums, both in a raw state and after being heated at various temperatures and periods. The modes of fixation were also in every case 1 hour in the water bath and 4 hours in the icebox.

These specific complement-fixing antibodies were found to be, without exception, persistently thermostable. In order to raise the coagulation point, the serums subjected to a temperature of 65 C. and higher were previously diluted 1:10 with salt solution.

<sup>5</sup> For preliminary report see Kahn, R. L.: *Proceed. Soc. for Exp. Biol. and Med.*, 1921, 18, p. 4.

<sup>6</sup> Kahn, R. L.: *Jour. Exper. Med.*, 1921, 34, p. 217.

Table 4 indicates that these antibodies are capable of withstanding a temperature of 70 C. for 15 minutes. The results of heating periods of 1 hour at 70 C. and a half hour at 75 C. have not been constant and for this reason are not recorded. At times there was practically no antibody destruction at these temperatures and periods and at other times there was about 25% or more antibody destruction. It would appear that the thermal destructive temperature for these antibodies lies between 70 C. and 80 C.

TABLE 3  
THE THERMOSTABILITY OF COMPLEMENT-FIXING ANTIBODIES PRODUCED BY PROTEIN  
IMMUNIZATION

Time and Temperature of Heating	Mode of Fixation	Serum of Rabbit Immunized with Edestin, C c									Total Plus Signs
		0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.0003	0.0001	
0	Water bath*....	2	2	1	1	—	—	—	—	—	6
	Icebox.....	3	3	1	1	—	—	—	—	—	8
5 min. at 56 C.	Water bath.....	3	3	1	1	1	—	—	—	—	9
	Icebox.....	4	4	2	2	—	—	—	—	—	12
15 min. at 56 C.	Water bath.....	3	2	1	1	1	—	—	—	—	8
	Icebox.....	4	4	2	2	—	—	—	—	—	12
30 min. at 56 C.	Water bath.....	3	3	1	1	—	—	—	—	—	8
	Icebox.....	4	4	3	2	—	—	—	—	—	13
60 min. at 56 C.	Water bath.....	3	3	1	1	—	—	—	—	—	8
	Icebox.....	4	4	3	2	—	—	—	—	—	13
15 min. at 62 C.	Water bath.....	3	3	1	1	—	—	—	—	—	8
	Icebox.....	4	4	3	2	—	—	—	—	—	13
30 min. at 62 C.	Water bath.....	3	3	1	1	—	—	—	—	—	8
	Icebox.....	4	4	3	2	—	—	—	—	—	13
60 min. at 62 C.	Water bath.....	3	3	1	1	—	—	—	—	—	8
	Icebox.....	4	4	3	2	—	—	—	—	—	13
30 min. at 65 C.	Water bath.....	3	3	1	1	—	—	—	—	—	8
	Icebox.....	4	4	3	2	—	—	—	—	—	13
60 min. at 65 C.	Water bath.....	3	3	1	1	—	—	—	—	—	8
	Icebox.....	4	4	3	2	—	—	—	—	—	13
120 min. at 65 C.	Water bath.....	3	3	1	1	—	—	—	—	—	8
	Icebox.....	4	4	1	1	—	—	—	—	—	10

\* The period of water bath fixation was 1 hour; of icebox fixation, 4 hours.

*Summary of Results Obtained With Protein Immune Serums.*—A study of the velocity of destruction of complement-fixing antibodies present in the serums of rabbits immunized with purified proteins showed these antibodies to be highly thermostable. Heating for 2 hours at 65 C. or for 15 minutes at 70 C. produced little effect on these antibodies. Prolonged heating at 70 C. and 75 C. resulted in varying degrees of antibody destruction. The tendency for stronger

binding of complement after a fixation period of 4 hours in the icebox compared with one hour in the water bath was noted also with these specific antibodies.

THE EFFECT OF HEAT ON COMPLEMENT-FIXING ANTIBODIES  
PRODUCED BY BACTERIAL IMMUNIZATION

The marked difference in the behavior toward heat of syphilitic complement-fixing substances compared with specific antibodies obtained after protein injections raised the question of the behavior toward this agent of similar antibodies obtained after bacterial

TABLE 4  
THE THERMAL DESTRUCTION OF SPECIFIC COMPLEMENT-FIXING ANTIBODIES

Immune Serum Rabbit	Protein Used in Immunization	Time and Temperature of Heating	Serum, C c									Total Plus Signs
			0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.003	0.0001	
1	Edestin	30 minutes at 56 C. ...	4	4	4	4	4	2	1	—	—	23
		15 minutes at 70 C. ...	4	4	4	4	4	1	1	—	—	22
		15 minutes at 80 C. ...	—	—	—	—	—	—	—	—	—	0
4	Edestin	30 minutes at 56 C. ...	3	2	1	1	—	—	—	—	—	7
		15 minutes at 70 C. ...	3	2	1	1	—	—	—	—	—	7
		15 minutes at 80 C. ...	—	—	—	—	—	—	—	—	—	0
A	Phaseolin	30 minutes at 56 C. ...	4	3	2	—	—	—	—	—	—	9
		15 minutes at 70 C. ...	4	2	1	—	—	—	—	—	—	7
		15 minutes at 80 C. ...	—	—	—	—	—	—	—	—	—	0
B	Phaseolin	30 minutes at 56 C. ...	4	4	4	4	4	4	1	—	—	25
		15 minutes at 70 C. ...	4	4	4	4	4	4	1	—	—	25
		15 minutes at 80 C. ...	—	—	—	—	—	—	—	—	—	0

Method of fixation, 4 hours at icebox temperature.

immunization. It was furthermore desired to determine to what extent the mode of fixation affected the velocity of thermal destruction of these antibodies.

The bacterial antigens employed were *B. typhosus*, *B. mallei* and *B. abortus*. The typhoid culture was obtained from the bacteriologic division of these laboratories and the antigen was prepared from 24 hour agar slants. The bacterial suspension was heated for 1 hour at

56 C. and 0.5% phenol added as a preservative. Cultures of *B. mallei* and *B. abortus* were kindly furnished us by Mr. I. F. Huddleson of the Bacteriological Department of the Michigan Agricultural College. Antigen suspensions of these organisms were prepared as in the case of *B. typhosus*. After regular antigenic titrations in the presence of their specific serums, these antigens were finally employed in such dilutions that 0.1 cc—the quantity used in the tests—contained 3 antigenic units and 0.3 cc were neither anticomplementary nor hemolytic.

TABLE 5

THE EFFECT OF THE MODE OF FIXATION ON THE VELOCITY OF THERMAL DESTRUCTION OF COMPLEMENT-FIXING ANTIBODIES PRODUCED BY IMMUNIZATION WITH *B. ABORTUS*

Time and Temperature of Heating	Temperature of Fixation (Period = 1 Hr.)	Immune Abortion Serum (Bovine), C c									Total Plus Signs
		0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.0003	0.0001	
0	Water bath.....	4	4	4	4	4	—	—	—	—	20
	Icebox.....	4	4	4	4	4	—	—	—	—	20
30 min. at 56 C.	Water bath.....	4	4	4	4	3	—	—	—	—	19
	Icebox.....	4	4	4	4	4	—	—	—	—	20
60 min. at 56 C.	Water bath.....	4	4	4	4	3	—	—	—	—	19
	Icebox.....	4	4	4	4	4	—	—	—	—	20
15 min. at 65 C.	Water bath.....	4	4	4	4	3	—	—	—	—	19
	Icebox.....	4	4	4	4	4	—	—	—	—	20
15 Min. at 70 C.	Water bath.....	4	4	4	3	2	—	—	—	—	17
	Icebox.....	4	4	4	3	2	—	—	—	—	17
15 min. at 75 C.	Water bath.....	2	1	1	1	1	—	—	—	—	6
	Icebox.....	2	1	1	1	1	—	—	—	—	6

With regard to the antisera, in the case of *B. typhosus* and *B. mallei*, rabbit serums were employed; in the case of *B. abortus*, bovine serum.

The complement-fixation tests were carried out in practically every detail as with the syphilitic and protein immune serums, except that the methods of fixation were 1 hour in the water bath and 1 hour (instead of 4 hours) in the icebox. Studies on the rate of fixation of complement with bacterial antisera are at present being carried out in this laboratory, and it was deemed best to employ a uniform period of 1 hour both at water bath and icebox temperatures.

Table 5 records the velocity of destruction of complement-fixing antibodies (bovine) obtained after immunization with *B. abortus*. It appears from this table that these antibodies begin to break down when subjected to a temperature of 70 C. The antityphoid and anti-glanders serums gave similar results. These experiments also indicated

that the phenomenon of fixation goes on equally well at water bath and icebox temperatures.

Table 6 gives the results of an experiment carried out with the three bacterial antisera. No antibody destruction was noted after heating these sera for 1 hour at 65 C. Heating for 1 hour at 70 C. showed variable degrees of destruction, and one-half hour at 75 C. was highly destructive to these antibodies.

TABLE 6  
THE THERMAL DESTRUCTION OF COMPLEMENT-FIXING ANTIBODIES PRODUCED BY  
BACTERIAL IMMUNIZATION

Immune Serum	Organism Used in Immunization	Time and Temperature of Heating	Serum, C c									Total Plus Signs
			0.01	0.007	0.004	0.003	0.002	0.001	0.0005	0.0003	0.0001	
Rabbit	B. typhosus	0.....	4	4	4	4	3	1	1	—	—	21
		60 minutes at 65 C. ...	4	4	4	4	3	1	1	—	—	21
		60 minutes at 70 C. ...	4	4	4	2	1	1	1	—	—	17
		30 minutes at 75 C. ...	4	3	2	1	1	1	—	—	—	12
		60 minutes at 75 C. ...	4	3	2	1	1	—	—	—	—	11
Rabbit	B. mallei	0.....	4	4	4	3	2	1	1	1	—	20
		60 minutes at 65 C. ...	4	4	4	3	2	1	1	1	—	20
		60 minutes at 70 C. ...	4	2	1	1	1	—	—	—	—	9
		30 minutes at 75 C. ...	2	1	1	1	—	—	—	—	—	5
		60 minutes at 75 C. ...	1	1	—	—	—	—	—	—	—	2
Bovine	B. abortus	0.....	4	4	4	4	4	4	4	2	2	32
		60 minutes at 65 C. ...	4	4	4	4	4	4	4	2	1	31
		60 minutes at 70 C. ...	4	4	4	4	4	4	2	1	1	28
		30 minutes at 75 C. ...	4	2	1	1	..	..	..	..	..	8
		60 minutes at 75 C. ...	2	1	..	..	..	..	..	..	..	3

Method of fixation, 1 hour at icebox temperature.

*Summary of Results Obtained With Bacterial Immune Sera.*—The velocity of destruction by heat of bacterial complement-fixing antibodies was investigated. The following antisera were employed: antityphoid (rabbit), antimallei (rabbit), and antiabortion (bovine). The results of complement-fixation studies conducted with raw sera and the same sera heated at different temperatures and periods indicated that these antibodies are quite as thermostable as those obtained on protein immunization. With regard to the mode of fixation,

the findings indicate that the difference between 1 hour fixation in the icebox from the same period in the water bath is not marked.

#### DISCUSSION

The observation that specific complement-fixing substances are capable of withstanding a temperature of 65 C. while so-called syphilitic antibodies are incapable of withstanding much lower temperatures is, in our opinion, significant. It places specific complement-fixing antibodies in a class with specific agglutinins and precipitins, and syphilitic complement-fixing substances apparently in a class by themselves. And yet, we question whether our data indicates that the complement-fixing antibodies in syphilis are essentially different from those found in other immune serums. Of the types of immune serums investigated, only those which were tested with nonspecific antigens showed the presence of thermolabile antibodies. Is it not likely, therefore, that the apparent thermolability of these antibodies is due to the nature of the antigens? This view is strengthened by the fact that both the mode of fixation and the type of antigen employed, markedly affect the complement binding power of heated syphilitic serums. In our opinion, in order to prove with reasonable certainty whether or not a given antibody is thermostable, the immune serum should be tested with a freshly prepared specific antigen.

On the other hand, this difference in the behavior toward heat by the two types of antibodies suggests that the phenomenon of complement fixation in syphilis is more or less distinct from complement fixation with specific antigens. And it is not unlikely that the negative complement-fixation findings in bacterial infections, such as tuberculosis and gonorrhea, in which a Wassermann procedure was employed, are inconclusive. For, the applicability of this procedure in syphilis does not necessarily imply its successful general application in other infectious diseases.

#### SUMMARY

It was shown that thermal destruction of syphilitic complement-fixing substances is markedly influenced by the mode of fixation. When fixation was carried out for 1 hour at water bath temperature, the heating of serum for 30 minutes at 56 C. showed in a total of 87 serums tested an average antibody destruction of 32%. When fixation was carried out for 4 hours at icebox temperature, some serums showed a slight loss, others, no loss and still others, a considerable gain in

antibody content, with the result that the average finding of the 87 serums tested represented a gain of 10%. Heating syphilitic serums for 20 minutes at 62 C. gave an average of 75% antibody destruction, with water bath fixation, and 46%, with icebox fixation.

The type of antigen employed was also found to influence thermal destruction of these antibodies. Heating serums for 30 minutes at 56 C. showed either little antibody destruction or an apparent gain in antibody content with 2 alcoholic extract antigens and one cholesterinized antigen and icebox fixation, while even with this mode of fixation, considerable destruction was noted at this temperature and period when employing the Noguchi antigen (table 2).

Finally, it was shown that complement-fixing antibodies obtained on protein or bacterial injections were comparatively thermostable. These antibodies were found to be capable of withstanding a temperature of 65 C. Temperatures of 70 C. and 75 C. showed varying degrees of antibody destruction—somewhat more so in the case of bacterial immune bodies than those obtained on protein injections.

With regard to the effect of the mode of fixation on specific antibody destruction due to heating, no marked difference was observed between water bath and icebox temperatures.



## STUDIES ON COMPLEMENT FIXATION

### IV. ON THE AFFINITY OF SHEEP CORPUSCLES FOR ANTISHEEP HEMOLYSIN \*

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A knowledge of the factors which affect the affinity of sheep corpuscles for antisheep hemolysin is of importance in all complement-fixation studies in which a sheep cell system is employed. It gives a measure of the rapidity of sensitization of sheep corpuscles in the presence of hemolysin. It indicates also quantitative means for the removal of so-called natural hemolysin from serums employed in complement-fixation tests. It was indeed this latter phase which led us to undertake this investigation. We had become convinced that the presence of antisheep hemolysin in some human serums interferes with the correctness of complement-fixation tests. It will be recalled that this source of error was one of the important factors which led Noguchi to devise the "human system" as a means for the diagnosis of syphilis. In discussing the results of a number of parallel tests which gave sharper results with the Noguchi as compared with the Wassermann procedure, this investigator<sup>1</sup> states: "Just how this difference in sharpness of reaction between my system and that of Wassermann arises has been repeatedly emphasized and there can be no doubt that this is due to the occasional excessive antisheep amboceptor present in some human sera under investigation." On the other hand, it is well known that in laboratories, where an average of 100 or more tests are examined daily, the sheep cell system is considered more practical than the Noguchi system. The original purpose, therefore, of this investigation was to study the affinity of sheep cells for antisheep hemolysin quantitatively with a view of finding whether a simple procedure could be devised for removing natural hemolysin from human serum without unduly delaying the completion of large numbers of Wassermann tests. Fortunately, several preliminary measurements of the rate of absorption of antisheep hemolysin by sheep cells, carried

Received for publication, July 29, 1921.

\*Preliminary Report, *Abst. of Bacteriol.*, 1921, 5, p. 24.

<sup>1</sup> *Serum Diagnosis of Syphilis*, Ed. 2, p. 122.

out by one of us,<sup>2</sup> suggested a simple and desirable method for hemolysin removal. The quantitative studies on the affinity of sheep cells for hemolysin were continued, however, and this paper will consider the following three phases: (1) the rate of absorption of hemolysin by sheep cells at different temperatures; (2) the effect of the concentration of hemolysin on the absorption capacity of a given amount of sheep cells; (3) the time and temperature of sensitizing sheep cells in the presence of hemolysin.

#### EXPERIMENTS

The hemolysin employed was prepared by immunizing rabbits with concentrated sheep cells, previously washed 5 times with normal salt solution. The animals were given 3 intravenous injections of packed cells at 48-hour intervals. The first injection consisted of 1 cc and the second and third of 2 cc each. Five days after the last injection, a hemolysin titer of 1:2,000 was usually obtained.

Complement was obtained by bleeding guinea-pigs under anesthesia directly from the heart. The blood was placed in the icebox immediately after clotting, and the clear serum was drawn off in about 15 hours after bleeding. From 4 to 5 pigs were bled at a time in order to insure the uniformity of the complement. No complement was used unless it was free from neutral hemolysin and possessed a good hemolytic titer.

The sheep cells were obtained from our own sheep kept for the purpose of supplying blood to the Wassermann laboratory. The cells were washed 4 times with salt solution and after the fourth, or final, washing, they were packed by centrifugation for 14 minutes at 1,500 revolutions per minute employing the same length of time and speed in each case. The concentrated cell suspension was employed for hemolysin extraction, while a 5% saline suspension was used in the hemolytic phase of these experiments. The latter is frequently spoken of as the standard sheep cell suspension.

Hemolysin considered in this paper is, in practically every case, expressed in units, and a unit was taken to be the smallest quantity which hemolyzed 0.1 cc of the standard sheep cell suspension in the presence of 0.1 cc of pooled 1:10 guinea-pig complement after 15 minutes' incubation in the water bath. This unit was determined by preparing a series of dilutions of hemolysin serum and titrating each

<sup>2</sup> Kahn, R. L.: *Jour. Lab. and Clin. Med.*, 1921, 6, p. 218

with 0.1 cc of the sheep cell suspension and 0.1 cc of pooled complement. These titrations were carried out in a series of 10 tubes, in the proportions shown in table 1.

The units were read after 15 minutes' incubation in the water bath, and the number of units contained in the undiluted hemolysin serum computed accordingly. Thus, if a serum dilution of 1:2,000 gave a titration unit of 0.05 cc, 1 cc of undiluted serum contained 40,000 units. (1 unit in 0.05 cc or 20 units in 1 cc;  $20 \times 2,000 = 40,000$ .)

TABLE 1  
PROPORTIONS IN WHICH TITRATIONS WERE CARRIED OUT IN TEN TUBES

Tubes	Hemolysin, C c	Complement (1:10) C c	Sheep Cells 5%, C c	Saline, Drops
1.....	0.1	0.1	0.1	3
2.....	0.09	0.1	0.1	3
3.....	0.08	0.1	0.1	3
4.....	0.07	0.1	0.1	3
5.....	0.06	0.1	0.1	4
6.....	0.05	0.1	0.1	4
7.....	0.04	0.1	0.1	4
8.....	0.03	0.1	0.1	5
9.....	0.02	0.1	0.1	5
10.....	0.01	0.1	0.1	5

1. THE RATE OF ABSORPTION OF HEMOLYSIN BY SHEEP  
CELLS AT DIFFERENT TEMPERATURES

To begin with, the rate of absorption of hemolysin by sheep cells at room temperature was studied. In the first absorption experiment, a dilution of hemolysin, each cc containing 20 units was employed. One cc quantities of this dilution were pipetted into 6 Wassermann tubes and 0.05 cc of packed sheep cells added to each. Extraction of hemolysin was permitted in the first tube for 5 minutes; in the second, for 10 minutes; in the third, for 15; in the fourth, for 20; in the fifth, for 25; and in the sixth tube for 30 minutes. Each tube was rapidly centrifuged at the end of its extraction period and the supernatant fluid drawn off.

Hemolysin titrations were carried out with the supernatant fluids of each of the 6 tubes in accordance with the following scheme:

Supernatant fluid (cc).....	0.5	0.3	0.1
Complement (cc).....	0.1	0.1	0.1
Sheep cells (cc).....	0.1	0.1	0.1

The results after 15 minutes' incubation in the water bath showed no hemolysis in any of the tubes in the quantities of supernatant fluid employed, indicating that the hemolysin originally contained in the solutions (20 units) was absorbed in every case.

This experiment was then repeated employing 1 c.c. quantities of hemolysin solution containing 100 units of hemolysin, with the same results. In other words, 0.05 c.c. of packed sheep cells extracted 100 units of hemolysin in 5 minutes at room temperature.

In the next step, six 1 c.c. quantities of hemolysin solution, each containing 200 units of hemolysin, were employed. Into each of these 6 tubes were added 0.05 c.c. packed sheep cells and, as in the previous experiments, extraction permitted for 5, 10, 15, 20, 25 and 30 minute periods. Each tube was centrifuged after its extraction period and the supernatant fluid again titrated in the presence of complement and sheep cells for unextracted hemolysin. The titrations were carried out in the same manner as indicated in the first experiment. It was found that 0.5 c.c. quantities of supernatant fluid of the tubes which underwent 5 minutes' extraction showed slight hemolysis after 15 minutes' incubation in the water bath. Half c.c. quantities of the supernatant fluids of tubes which received longer extraction periods showed, in each case, a trace of hemolysis, only.

Our next problem was to find whether the same amount of packed cells added to 1 c.c. human serum containing 200 units of hemolysin would extract this quantity in this short period. This was of importance, in view of the fact that in complement-fixation tests the problem involved is the removal of natural hemolysin from serum. Accordingly, 0.01 c.c. of undiluted hemolysin containing 200 units was added to 1 c.c. quantities of pooled human serum and extractions carried out with 0.05 c.c. quantities of packed sheep cells at 5, 10, 15, 20, 25 and 30 minute intervals. The human serum was obtained by mixing serums sent to this laboratory for Wassermann tests, after completing the examinations. It was found that the supernatant fluids obtained on centrifugation after these extraction periods were free from hemolysin, indicating extraction of the 200 hemolysin units originally contained in these solutions.

Having shown that 0.05 c.c. of packed sheep cells were capable of absorbing close to 200 units of hemolysin from saline solutions and the same number from serum solutions after short extraction periods at room temperature, our next problem was to find to what extent

the temperature affects hemolysin extraction, and, also, whether the cells from different sheep show variations in the amount of hemolysin extracted. It was also decided to use 400 instead of 200 units of hemolysin. The extraction periods in these experiments were 5, 10, 15 and 20 minutes, and the temperatures were water bath (37.5 C) room (18-24 C.) and icebox (8-12 C.).

The protocol of the first experiment is herewith given in detail:

A hemolysin solution was prepared in the proportion of 0.01 cc undiluted hemolysin to 1 cc of salt solution. One cc of this solution contained 400 units, determined by a hemolysin titration according to the procedure indicated in the foregoing. One cc quantities of this hemolysin solution were added to a series of 12 tubes. The packed sheep cell suspension was then prepared by

TABLE 2  
EXPERIMENT 1

Time of Adding Sheep Cells	Extraction Temperatures											
	Room (21 C.)				Water Bath (37.5 C.)				Icebox (10 C.)			
	Cell Suspension				Cell Suspension				Cell Suspension			
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 1	Tube 2	Tube 3	Tube 4	Tube 1	Tube 2	Tube 3	Tube 4
9:00 a. m. ....	C c	C c	C c	C c	C c	C c	C c	C c	C c	C c	C c	C c
9:05 a. m. ....	...	...	...	0.05	...	...	...	0.05	...	...	...	0.05
9:10 a. m. ....	...	...	0.05	...	...	...	0.05	...	...	...	0.05	...
9:15 a. m. ....	...	0.05	...	...	...	0.05	...	...	...	0.05	...	...
9:20 a. m. ....	0.05	...	...	...	0.05	...	...	...	0.05	...	...	...
9:20 a. m. ....	All tubes were centrifuged at high speed and supernatant fluid drawn off											

washing 4 times with salt solution in the usual manner and centrifuging for 14 minutes at 1,500 revolutions per minute; 0.05 cc quantities of this suspension were then added at different intervals to each of the 12 tubes. These intervals were so arranged as to give each series of 3 tubes an extraction period of either 5, 10, 15 or 20 minutes, in accordance with table 2. (Each tube contained 1 cc hemolysin solution containing 400 units.)

In order to find the number of hemolysin units extracted in each case, a regular hemolysin titration was carried out with each of the supernatant fluids. The readings of these hemolysin titrations, and the computed number of hemolysin units extracted by the sheep cells at different periods and temperatures is given in table 3.

The computation of the number of hemolysin units extracted at each of the temperatures was a relatively simple matter. Thus, in the case of supernatant fluid 1 (room temperature) and its unit of 0.03, the number of units extracted by the sheep cells was obtained in the

following manner: 1 unit of hemolysin is contained in 0.03 c c; 33.3 units of hemolysin are contained in 1 c c; originally 1 c c contained 400 units;  $400 - 33.3 = 366.7$ .

The experiment just described was repeated in every detail with pooled human serum instead of salt solution. This was prepared by adding undiluted hemolysin to serum in the proportion of 0.01 c c to 1 c c of serum. Each c c in this case also contained 400 units of hemolysin. The results obtained with these serum solutions were similar to those obtained with the salt solutions, except that there were a somewhat larger number of units extracted from the serum compared with the salt solution.

TABLE 3  
RESULTS OF HEMOLYSIN TITRATION

Tubes	Temperature of Extraction	Time of Extraction in Minutes	Titration Readings, C c	Number of Hemolysin Units Extracted
1	Room.....	5	0.03	367
	Water bath.....	5	0.02	350
	Icebox.....	5	0.02	350
2	Room.....	10	0.03	367
	Water bath.....	10	0.03	367
	Icebox.....	10	0.02	350
3	Room.....	15	0.03	367
	Water bath.....	15	0.03	367
	Icebox.....	15	0.02	350
4	Room.....	20	0.03	367
	Water bath.....	20	0.03	367
	Icebox.....	20	0.02	350

These hemolysin extraction experiments carried out in each case with salt and serum solutions were repeated 11 times, using the corpuscles of 5 different sheep. The cells of 1 sheep were employed in 3 experiments; the cells of each of the others, in 2 experiments.

In view of the fact that the number of hemolysin units extracted in each of these experiments closely approximate one another, the table representing the results of each of the 11 experiments is not given. Instead, the average findings of these experiments are presented.

The corpuscles from the 5 different sheep did not show any marked variation in their hemolysin absorption capacity. It will be noted that the extraction at water bath temperature was greater than at room temperature and this, in turn, greater than at icebox temperature. But the differences were comparatively small. Neither were there marked differences in hemolysin absorption between 5 and 20 minutes.

It might be added also that in some of these experiments, ordinary sized drops of packed cells were employed instead of 0.05 c c quantities. This was done because in the procedure for removing natural hemolysin, proposed by one of us,<sup>2</sup> the addition of drops of packed sheep cells per 1 c c quantities of serum is recommended. It was desired, therefore, to establish that practically the same results will be obtained with a drop as with 0.05 c c of packed cells.

## 2. THE EFFECT OF THE CONCENTRATION OF HEMOLYSIN ON THE ABSORPTION CAPACITY OF 0.05 CC OF PACKED SHEEP CELLS

There seems to exist much difference of opinion among investigators as to the amount of hemolysin a given quantity of cells is capable of absorbing in a given time. This becomes evident when

TABLE 4  
RATE OF ABSORPTION OF ANTISHEEP HEMOLYSIN BY SHEEP CORPUSCLES AT DIFFERENT TEMPERATURES

Time of Extraction, Minutes	Number of Units of Hemolysin Extracted from a Solution of 400 Units at					
	Water Bath Temperature from		Room Temperature from		Icebox Temperature from	
	Salt Solution	Serum Solution	Salt Solution	Serum Solution	Salt Solution	Serum Solution
5	376*	399	363	380	356	361
10	382	399	377	388	370	371
15	390	400	384	392	376	377
20	388	400	387	395	379	379

\* Each number represents an average of 11 different extraction experiments.

one considers the time and temperature for sensitization of sheep cells, as recommended in three recently standardized Wassermann procedures. Thus Neil<sup>3</sup> of the Hygienic Laboratory recommends mixing the sheep cells with hemolysin and letting the mixture remain for 15 minutes at room temperature; Hinton,<sup>4</sup> for 1/2 hour at 37 C.; and Kolmer,<sup>5</sup> for 1 hour at room temperature. That sheep corpuscles are capable of absorbing huge quantities of specific hemolysin has been shown by Morgenroth and Arrhenius.<sup>6</sup> It seemed advisable, however, to attempt to find the absorption capacity of 0.05 c c of packed sheep cells when exposed to different concentrations of hemolysin for 10 minutes at room temperature. This quantity of sheep cells was chosen

<sup>3</sup> Public Health Reports, 1918, 33, p. 1387.

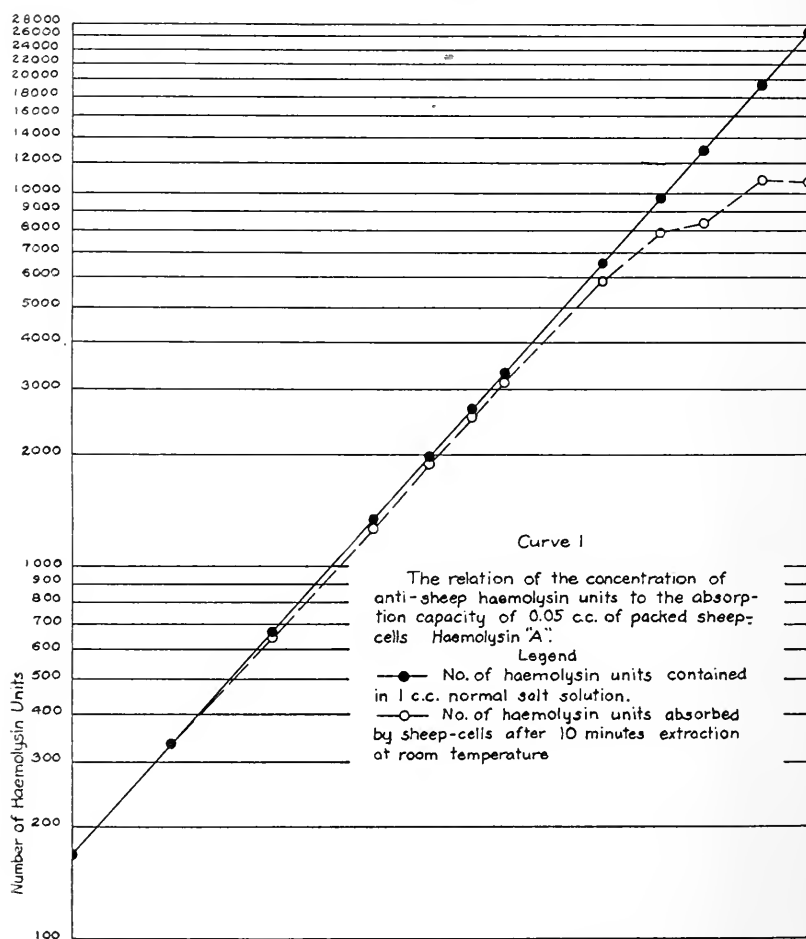
<sup>4</sup> Jour. of Syph., 1920, 4, p. 598.

<sup>5</sup> Ibid., p. 616.

<sup>6</sup> Quoted by Arrhenius, S.: Immunochemistry, p. 150.

because it is equivalent to 1 cc of a 5% suspension—a quantity which represents the amount of cells employed in the regular Wassermann procedure.

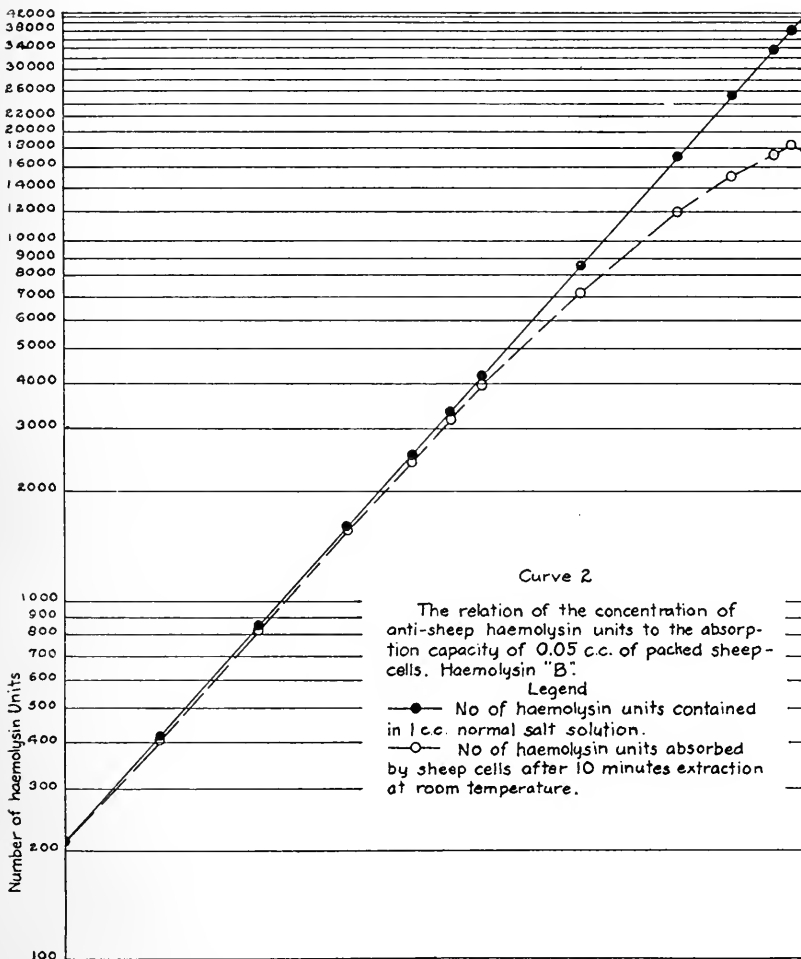
Ten different stock solutions of antish sheep hemolysin were used in these experiments. The general plan was to pipet various gradations



of hemolysin serum into a series of Wassermann tubes. These gradations ranged from 1 cc to 0.01 cc. To those which contained less than 1 cc, sufficient salt solution was added to make up to this quantity. To each tube was then added 0.05 cc of packed sheep cells, carefully mixed and allowed to remain for 10 minutes at room temperature. The



tubes were then centrifuged at high speed and the supernatant fluid drawn off. Regular hemolysin titrations were carried out before subjecting the serum to the sheep cells, and with the supernatant fluids after extraction, to determine in each case the number of hemolysin



units per c.c and thus determine the number of units extracted by the corpuscles.

The results of two such experiments are given in table 5. To what extent the number of units of hemolysin absorbed depends on the concentration of hemolysin is particularly well illustrated in charts 1 and 2.

It will be noted that the number of hemolysin units are indicated on an arbitrary line and the number of absorbed units are diverging from this line little by little until 10,000 or more units are reached. The main reason why the absorption lines do not follow the original lines to the end is because of the marked agglutinating power of undiluted (or little diluted) hemolysin serum on sheep cells. Thus, when 0.05 c c of packed sheep cells were added to 1 c c of hemolysin (serum A), the corpuscles went to the bottom of the tube in a few seconds. The effect of these hemagglutinins was to prevent the corpuscles from being in continuous contact with the hemolysins even on frequent shaking. The lack of constancy in results, therefore, is what one might have expected.

TABLE 5  
THE EFFECT OF THE CONCENTRATION OF HEMOLYSIN ON THE ABSORPTION CAPACITY OF 0.05  
C C OF PACKED SHEEP CORPUSCLES AFTER EXTRACTION FOR 10 MINUTES  
AT ROOM TEMPERATURE

Hemolysin A				Hemolysin B			
Tubes	Number of Hemolysin Units per C c	Number of Units Remaining after Extraction	Number of Units Extracted	Tubes	Number of Hemolysin Units per C c	Number of Units Remaining after Extraction	Number of Units Extracted
1	166	0	166	1	212	0	212
2	333	0	333	2	425	3	422
3	666	10	656	3	850	11	839
4	1,332	33	1,299	4	1,700	33	1,667
5	1,998	75	1,923	5	2,550	109	2,441
6	2,664	125	2,539	6	3,400	200	3,200
7	3,330	200	3,130	7	4,250	266	3,984
8	6,660	530	5,830	8	8,500	1,250	7,250
9	9,990	2,000	7,990	9	17,000	5,000	12,000
10	13,320	5,000	8,320	10	25,500	10,000	15,500
11	19,980	9,180	10,800	11	34,000	20,000	14,000
12	26,640	16,000	10,640	12	38,250	20,000	18,250
				13	42,500	25,000	17,500

It will be noted also that in view of the large range of hemolysin units employed, logarithmic paper was used in making these curves.

A consideration of the laws which govern the absorption of anti-sheep hemolysin by sheep cells is reserved for further studies. This, however, is established, namely, that 0.05 c c of packed sheep corpuscles are capable of absorbing as many as 18,000 units of hemolysin after 10 minutes' extraction at room temperature.

### 3. THE TIME AND TEMPERATURE OF SENSITIZING RED CELLS IN THE PRESENCE OF HEMOLYSIN

The foregoing studies suggested still another phase in connection with the union of sheep cells and hemolysin. We have in mind the

element of dissociation. Thus, it was observed that when 0.05 c c quantities of packed sheep cells were added to 1 c c solutions of hemolysin, each containing 400 units, more units were absorbed in some cases after 10 minutes' extraction at room temperature than after one hour extraction at water bath temperature. More frequently, the number of units absorbed after 10 minutes' extraction at room temperature was equal to the number of units absorbed after 1 hour in the water bath. The results of two such experiments are given in table 6.

There are indeed two possible factors which are liable to interfere with the absorption of hemolysin by sheep cells after an exposure of 1 hour in the water bath: first, the probable element of dissociation

TABLE 6  
THE EFFECT OF TIME AND TEMPERATURE ON THE HEMOLYSIN ABSORPTION CAPACITY OF  
0.05 c c PACKED SHEEP CELLS

Hemolysin C					Hemolysin D				
Tubes	Number of Units per C c	Temperature of Extraction	Time of Extraction, Min.	Units Extracted	Tubes	Number of Units per C c	Temperature of Extraction	Time of Extraction, Min.	Units Extracted
1	9,900	Water bath	10	6,660	1	7,500	Water bath	10	5,190
2	9,900	Room	10	6,240	2	7,500	Room	10	5,190
3	9,900	Water bath	60	6,240	3	7,500	Water bath	60	4,800
4	9,900	Water bath	120	6,240	4	7,500	Water bath	120	4,420

during this period; second, the possibility of hemolysis of a small number of corpuscles, liberating thereby a small quantity of hemolysin. A trace of hemolysis was indeed observed in hemolysin 2 (table 3) after centrifuging the tubes which were kept in the water bath 1 hour and longer.

#### SUMMARY

A quantitative study of some factors which govern the affinity of sheep corpuscles for antisheep hemolysin was carried out. The hemolysin was obtained by immunizing rabbits with sheep corpuscles in the usual manner. The corpuscles employed were obtained interchangeably from 5 different sheep. A unit of hemolysin was taken to be the smallest quantity which completely hemolyzed 0.1 c c of a 5% suspension of sheep cells in the presence of 0.1 c c of pooled guinea-pig complement after 15 minutes' incubation in the water bath.

In the first series of experiments, the rate of absorption of anti-sheep hemolysin by sheep corpuscles at different temperatures was

studied. The extraction periods were 5, 10, 15 and 20 minutes, and the temperatures were water bath, room and icebox. The hemolysin was extracted from both salt and pooled serum solutions. It was found, when 0.05 c c quantities of packed sheep corpuscles were added to 1 c c quantities of either salt or serum solutions, each containing 400 units of hemolysin, that the differences in the quantity of hemolysin absorbed at extraction periods of 5 to 20 minutes were not marked. Neither were there large differences in the number of units absorbed at water bath and icebox temperatures (table 1).

In the second series, the effect of the concentration of hemolysin on the absorption capacity of 0.05 c c of packed sheep cells was studied. The extraction was in each case carried out for 10 minutes at room temperature. It was shown that the number of hemolysin units that this quantity of sheep corpuscles is capable of absorbing is directly proportional to the concentration of hemolysin. The largest number of units that 0.05 c c of packed cells absorbed in these experiments was 18,000. This number, however, does not represent the true absorption capacity of this quantity of sheep cells. The hemolysin serums employed, either undiluted or in low dilution, contained in every instance large numbers of hemagglutinins. These tended to bring about immediate precipitations of the corpuscles and thereby prevented proper contact between the hemolysin and the cells.

Finally, the effect of time and temperature on the hemolysin absorption capacity of 0.05 c c of packed sheep cells was studied. It was found that a 10 minute extraction period at room temperature was in most cases equivalent to a 1 hour or 2 hour extraction period at water bath temperature. In a few cases, there was less extraction after 1 hour or 2 hours in the water bath compared with 10 minutes' extraction at room temperature. This is believed to be due to dissociation of hemolysin and cells after prolonged extraction at 37.5 C. and also to the hemolysis of some corpuscles at this temperature with the liberation of some hemolysin.

# THE PATHOGENICITY OF *B. ABORTUS* AND *B. MELITENSIS* FOR MONKEYS

STUDIES ON THE GENUS *BRUCELLA* NOV. GEN. III.

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Recent observations made by A. C. Evans,<sup>1</sup> by Meyer and Fleischner, Shaw and Feusier<sup>2</sup> and by Zeller<sup>3</sup> have proven that *B. abortus* and *B. melitensis* are morphologically, biochemically and serologically closely related. In order to demonstrate conclusively the relationship of the two bacteria, it becomes necessary to conduct infection experiments on animals. In several series on guinea-pigs, it has been demonstrated that the lesions produced in the spleen, the lymph nodes, etc., following an intratesticular inoculation of certain *B. melitensis* strains, are indistinguishable from those produced by *B. abortus*. As the majority of the experiments by early observers on *B. melitensis* has been carried out on the monkey, an animal which, as originally shown by Bruce<sup>4</sup> and afterward by Hughes,<sup>5</sup> can be easily and certainly infected, it has seemed advisable to make similar experiments with *B. abortus*. The various reports of the Commission for the Investigation of Mediterranean Fever record numerous experiments dealing with infections produced in monkeys by subcutaneous, intramuscular and intravenous inoculations. Even the feeding of a single sample of infected goat's milk to 28 monkeys caused in 26 of them a disease which was very similar to that seen in man. It is stated that, as a rule, a *B. melitensis* infection of a monkey can be judged by the animal's temperature, which may be intermittent or remittent in character, by the appearance of specific agglutinins in the blood serum, and by slow, but definite loss in body weight.

As soon as the close relationship of *B. abortus* to *B. melitensis* had been established, it was manifestly important to determine experi-

Received for publication Aug. 1, 1921.

<sup>1</sup> Jour. Infect. Dis., 1918, 22, p. 580.

<sup>2</sup> Proc. Soc. Exper. Biol and Med., 1919, 16, p. 151; Jour. Infect. Dis., 1920, 27, pp. 173 and 185.

<sup>3</sup> Berl. tierarztl. Wchnschr., 1920, 36, p. 345.

<sup>4</sup> Ann. de l'Institut. Pasteur, 1893, 7, p. 289.

<sup>5</sup> Lancet, 1892, 2, p. 1265.

mentally the following question: Can *B. abortus* produce in the monkey a similar syndrome as *B. melitensis*? Careful review of the work that had been done with *B. abortus* showed that only one investigator used monkeys. Marshal Fabyean,<sup>6</sup> in his studies on the persistence of this organism in the tissues of inoculated animals, infected two monkeys and was able to recover *B. abortus* 12 and 13 weeks, respectively, after the inoculation. He did not state the method used in inoculating the animals and gave no additional observations as to the results.

Aside from reproducing an undulant fever-like disease by direct inoculation of *B. abortus*, it was obviously important to determine carefully the possibility of causing an infection by feeding either cultures or goats milk containing the bacteria in small or large numbers. These experiments led to a consideration of the so-called "virulence" of *B. abortus* and resulted in a number of similar experiments with strains of different origin. It seemed also possible that more definite knowledge might be obtained concerning the pathogenicity of *B. abortus* for children and adults, if a study of feeding infections in monkeys were undertaken. The experiments reported here, therefore, were planned to supply a number of facts, which would assist in answering the question in a somewhat more intelligent manner than had hitherto been possible.

In order to be familiar with the temperature reactions, and the symptoms produced in monkeys by the organisms of malta fever, a number of infection experiments were made with certain interesting strains of *B. melitensis*. These cultures had been found to be pathogenic for guinea-pigs, and quite frequently they could not be differentiated from those of true abortion bacilli.

#### METHODS

Most of the experiments were made on young monkeys and if possible on one species, namely, *Pithecus syrichta*, imported from India. All were fresh stock and were brought directly to the laboratory on their arrival by steamer in San Francisco. On two occasions two old stock animals, a *Macacus cynomolgus* and a *Cercopithecus philippinensis* were also used. The animals were kept in single cages and special care was taken to avoid cross infection by contaminated food, urine, etc. This precaution was particularly necessary in connection with the *B. melitensis* experiments.

<sup>6</sup> Jour. Med. Res., 1913, 28, p. 82.

The organisms used for the tests were grown on glycerol peptic digest agar slants (PH 7.0) at 37 C. for 2-7 days. The monkeys were fed by either smearing the bacterial growth on carrots or by suspending the cultural growth in 50-100 c c of milk. The latter was offered to the starved animals in small pannikins. As a rule, the monkeys drank the milk without wasting more than 20-30 c c. Blood stream inoculations were made by way of the jugular or saphenous vein.

The methods of study after inoculation or feeding consisted in the observation and record of clinical symptoms, the taking of morning temperature (rectal), repeated agglutination tests with the blood serum, and hemoglobin determinations. Formalinized suspensions of *B. abortus* and *B. melitensis* as specified in previous papers were used as antigens for the agglutination tests. Necropsies with bacteriologic examinations were made on all animals that died or were

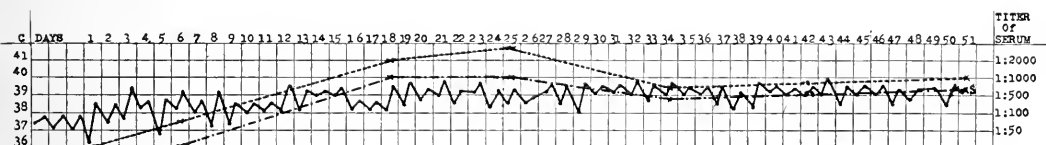


Chart 1 (Monkey 2).—Intravenous inoculation of *B. abortus*.

killed. Cultures of the tissues were made on blood agar plates, glycerol peptic digest agar slants and glycerol veal infusion broth. The strains isolated were identified by agglutination and absorption tests, as detailed in paper II of this series.

#### EXPERIMENTAL DATA

##### *Infections Produced by the Intravenous Inoculations of B. abortus.*

—Three normal monkeys were injected intravenously with typical abortion bacilli in amounts ranging from 2-16 billion of a glycerol peptic digest agar culture. The following protocols detail the various observations.

*Exper. 1.—Monkey 2.*—*Pithecus syrichta*, female, weight 1,875 gm. March 18, to April 3, 1919, well and active; serum repeatedly examined for *B. abortus* and *B. melitensis* agglutinins with negative results. April 3, 3:00 p. m., inoculated intravenously in left jugular vein  $\frac{1}{2}$  slant of a 48-hour old peptic digest agar slant—2 billion of *B. abortus* 80 (origin of culture—certified milk—guinea-pig passage, 2½ years old); bled from the right ear 15 minutes after injection.

Blood smeared on agar slant gave profuse growth of *B. abortus*. The temperature during the period of observation of 51 days was always above 39 C. (chart 1).

April 24, the animal developed diarrhea which lasted for 2 days. Blood cultures on the sixth and eighteenth day remained sterile. The blood gave the following agglutination reactions:

April 9, or 6 days after injection—*B. abortus* 80=1:80; *B. melitensis* 11=0.

April 21, or 18 days after injection—*B. abortus* 80=1:2000; *B. melitensis* 11=1:1000.

April 28, or 25 days after injection—*B. abortus* 80=1:4000+; *B. melitensis* 11=1:1000.

May 7, or 34 days after injection—*B. abortus* 80=1:600 (inhibition zone 1:200); *B. melitensis* 11=1:400.

May 24, 1919, or 51 days after injection—*B. abortus* 80=1:1000 + + +; *B. melitensis* 11=1:600 + + +.

Skin tests conducted May 7 and 22 with abortus and melitensis protein were negative. May 24, the animal was chloroformed and cultures carefully made, 51 days after the intravenous infection.

*Postmortem Examination.*—The cadaver was slightly emaciated and weighed 1,680 gm., a loss of 200 gm. The spleen was normal in size and light in color, and the lymph nodes were distinctly enlarged and rather firm. The liver, kidneys, and lungs appeared normal. A small superficial ulceration was found on the mucous membrane of the rectum. This lesion was probably caused by the careless insertion of the thermometer. Numerous trichocephalus worms were noted in the appendix and colon. Cultures were made from the heart blood, spleen, liver, kidneys, bile, mesenteric, mediastinal, femoral and axillary lymph nodes, bone marrow, lungs and urine. The agar slants inoculated with liver, spleen, right and left kidneys and femoral lymph nodes were densely crowded with colonies of *B. abortus*.

*Exper. 2.—Monkey 11.*—*Pithecus syrichta* (Linn.), male, weight 2180 gm. Agglutination tests repeatedly were negative to *B. abortus* and *B. melitensis*. Aug. 6, 1919, at 2:00 p. m., the animal was inoculated intravenously with ½ slant or 16 billion *B. abortus* No. 14 (isolated from a fetal membrane of a cow in 1917). The monkey never suffered from fever and was always active. The following serum reactions were recorded during the course of the infection:

Aug. 21, or 15 days after injection:

*B. abortus* 80=1:10—1:100—0.

1:200—1:2000 + + +.

*B. melitensis* 2=1:10—1:60—0.

1:80—1:800 + + +.

*B. melitensis* 5=1:40—1:6000 + + +.

*B. melitensis* 3, 6 and 1=no agglutination.

Sept. 20, 1919, or 45 days after the injection:

*B. abortus* 80=1:2000 + + +, clumps.

*B. abortus* 14=1:40—1:80 neg. 1:100—1:1000 + + +.

Nov. 12, or 98 days after the infection:

*B. abortus* 80=1:80 + + +.

*B. melitensis* 655, 11 and 614=all negative.

In November, 1919, about 3 months after the intravenous injection of the abortus bacilli, the animal became suddenly ill and was found dead in his cage Nov. 12, 1919.



*Postmortem Examination.*—Body emaciated, weight 1,750 gm.; spleen small and firm; mesenteric lymph nodes slightly enlarged; tissues rather atrophic and dry; cause of death not determined.

Cultures from heart blood, liver, spleen, kidneys, lymph nodes and bone marrow contained numerous cocci and rods, but no *B. abortus*.

*Expcr. 3.—Monkey 12.*—*Pithecus syrichta* (Linn.); female, weight, 1,960 gm. Repeated agglutination tests with serum were negative for *B. abortus* and *B. melitensis*. Inoculated Aug. 14, 1919, intravenously with  $\frac{1}{4}$  slant or 12 billion *B. abortus* No. 80 isolated from liver of monkey 2. Temperatures on Aug. 15, 16, 17 and 18, normal. Monkey found dead at 4:00 p. m. Aug. 18, the fourth day after the injection.

*Postmortem Examination.*—Body well nourished; considerable fluid in abdominal cavity; spleen enlarged and rather soft; liver and kidneys congested; intestines omentum and mesenteric folds injected; lymph nodes in general slightly enlarged and soft. Heart muscle rather flabby, slight ventricular dilatation. Death was probably due to cardiac insufficiency.

Cultures were made from the heart blood, spleen, bone marrow, kidneys, bile, mesenteric, femoral and axillary lymph nodes. *B. abortus* was recovered from all the cultures except those made with heart blood and bile. The blood plates were densely crowded with colonies.

Exper. 1 must be considered in every respect successful; the monkey reacted to an intravenous injection of a moderately virulent strain of *B. abortus* by a marked production of specific agglutinins. The temperature curve, which represented the remittent type of pyrexia, resembled in many respects that reported on monkey 47 in Horrock's studies<sup>7</sup> on the conveyance of *M. melitensis* to healthy animals. The type of temperature was comparable to the one obtained in man, when the patient was suffering from what Shaw designated the "ambulatory" type of Mediterranean fever. The persistence of the infection, which in part explained the high agglutinin titer on the 51st day was proved by the extensive growth of *B. abortus* on the cultures prepared from the various organs. The observations on monkey 11, from a serologic standpoint, were similar to those noted in monkey 1. The infection exhibited itself primarily by an active production of agglutinins, although the temperature remained normal and the tissues on the 98th day after the injection were found to be free from *B. abortus*. The negative cultural finding of the tissues after death might in part be the result of the partial overgrowth of the plates and tubes by a coccus, which was probably responsible for the intercurrent disease. In view of the progressively diminishing serum agglutination reached before death, it appeared, however, possible that the monkey had recovered from the infection. Exper. 3 should be repeated in order to prove or disprove our conception that monkey 12 succumbed to a nonspecific injury to the heart muscle provoked by an intravenous inoculation of bacterial protein.

<sup>7</sup> Report of the Commission on Mediterranean Fever, London, 1905, Part I, p. 50.

The experiments definitely indicate that monkeys are susceptible to *B. abortus* after intravenous inoculations and that these animals may show febrile reactions similar to those observed in monkeys infected with *B. melitensis*. The injection calls forth a noteworthy production of serum agglutinins and the bacteria persist for at least 50 days in the liver, spleen, kidneys and lymph nodes, while the blood stream sterilizes itself rapidly.

*Infections Produced by the Feeding of Carrots, Bread, or Apples Contaminated with B. abortus of Low Virulence.*—In order to prove some of the questions raised in the introduction, it was necessary to make certain experiments by introducing *B. abortus* into the body via the alimentary tract. Three problems suggested themselves in this connection, namely: 1. Was the single ingestion of a limited number of bacteria sufficient to cause an infection? 2. If not, what was the approximate number of infective feedings and quantity of bacteria necessary to produce a disease similar to Malta fever? 3. What were the evidences of a successful feeding infection? It should be borne in mind in studying this problem with *B. abortus*, that, according to Eyre, McNaught, Kennedy and Zammit,<sup>7</sup> a *B. melitensis* infection, produced in the monkey by feeding, could not always be ascertained by the course of the animal's temperature or by its general appearance and behavior. In many instances, the postmortem findings and the demonstration of innumerable Malta fever organisms in the tissues rudely contradicted the negative clinical or even the serologic observations.

Six monkeys were fed with *B. abortus* cultures; 5 received the growth of a strain of low virulence, while 1 ingested a highly pathogenic culture of porcine origin. The method of administration of the infective material was quite simple; the bacterial masses of well-grown cultures were smeared on sliced carrots, bread or apples and immediately offered to the animals, by whom the contaminated food was eagerly eaten. The following protocols detail the various experiments:

*Exper. 4.—Monkey 1.*—*Pithecus syrichta* (Linn.) male; weight, 2,625 gm. March 18 to April 2, 1919, well and active, serum repeatedly examined for *B. abortus* and *melitensis* agglutinins with negative results. April 3, 3:30 p. m., fed the growth of 2 peptic digest agar slants of a 48-hour old culture of *B. abortus* 752 (about 25 billion organisms) smeared on a sliced carrot. The monkey never suffered from fever and the serum reactions conducted on April 20, 28; May 7, 22; June 10 and 25, and July 8, failed to reveal specific agglutinins. On July 20 the animal developed severe diarrhea and was chloroformed in extremis on July 23, or 110 days after feeding the culture.

*Postmortem Examination.*—Body emaciated, weight 2,025 gm., catarrhal enteritis, enlarged mesenteric lymph nodes, slight spleen tumor and congestion of liver. Cultures were negative for *B. abortus*, but there was an extensive growth of various intestinal organisms from liver, spleen, lymph nodes and bone marrow. The heart blood was sterile.

The blood serum collected at necropsy agglutinated *B. abortus* 320 in a dilution of 1:10.

This experiment indicated that a single feeding of a fairly large dose of virus failed to produce an infection in the monkey. It was considered unnecessary to repeat this experiment as similar results were obtained in animals fed with artificially infected milk (exper. 11 and 12).

*Exper. 5.—Monkey 3.*—*Pithecus syrichta*, male, weight, 2,280 gm. Had been used in exper. 13, in which he had received repeated feedings of goat's milk containing large numbers of *B. abortus*, with negative results; blood serum repeatedly negative for *B. abortus* and *B. melitensis*. From Oct. 4 to Nov. 10, 1919, the monkey was fed daily the growth of one slant each of *B. abortus* 80 and 14, or an approximate total of 2,270 billion bacteria smeared on sliced carrots or bread. The serum tested Oct. 25, the twentieth day of feeding, was negative. The temperature was normal until Nov. 10, when it rose to 41.5 C. and remained at this level 24 hours. On the morning of Nov. 12, the 36th day of the experiment, the monkey was found dead.

*Postmortem Examination.*—The body was well nourished; mucous membranes were slightly anemic. Lungs showed numerous bronchopneumonic areas. The spleen was normal in size and consistency. Liver, kidneys, mesenteric, femoral and subscapular lymph nodes were normal.

Cultures of the lungs gave a pure growth of *B. bronchisepticus*; the lymph nodes and liver showed streptococci; while the blood plates smeared with the spleen pulp were covered with numerous typical *B. abortus* colonies together with a few streptococcus colonies. The heart blood was sterile, but the serum agglutinated *B. abortus* 80 in a dilution of 1:600 + + + and *B. abortus* 14 in a dilution of 1:800.

*Exper. 6.—Monkey 13.*—*Ceropithecus philippinensis*, female, weight, 2,225 gm. Aug. 5, 1919, well and active; serum negative for *B. abortus* and *B. melitensis*. Fed on August 5, 6, 7, 8, 9, 10, 11, 13, 14, 21, 24 the growth of one slant each of *B. abortus* 80 and 14 on carrots, bread or apples. An approximate total of 1,300 billion organisms were fed. Marked loss in weight. Temperature was never above 40 C.; the blood serum gave the following reactions:

First day of feeding—*B. abortus* 80=0.

*B. melitensis* 2=0.

Weight 2,225 gm.

Sixteenth day of feeding—*B. abortus* 80=1:40 + + +, 1:80 + +.

*B. melitensis* 2=0.

Twenty-second day of feeding—*B. abortus* 80=1:80 + + +.

*B. melitensis* 11=1:100 + + +.

Weight 1,965 gm.

*B. abortus* 14=1:100 + + +.

*B. melitensis* 655=1:100 + + +.

Weight 1,890 gm.

Aug. 26, had severe diarrhea and refused to eat. Aug. 27, no improvement; chloroformed.

*Postmortem examination.*—The cadaver was emaciated; 5 c.c. of fluid were found in the abdominal cavity; the mesenteric and the femoral lymph nodes were enlarged and rather soft. The spleen was enlarged, the pulp soft with enlarged follicles. The kidneys, the liver and lungs appeared normal. Numerous oesophagostomum nodules were present in the colon and cecum.

Cultures made from the liver, kidneys, urine, lung, bone marrow, femoral, iliac and mediastinal lymph nodes were sterile or contained a few cocci colonies. The plates smeared with the mesenteric lymph nodes were densely covered by typical colonies of *B. abortus*. The agar slants and the blood plates seeded with spleen pulp showed 11-20 colonies of *B. abortus*.

*Exper. 7.*—*Monkey 33.*—*Pithecius syrichta* (Linn.), male; weight, 2,575 gm. Agglutination tests with *B. abortus* 80; *B. melitensis* 7 and 11 were repeatedly negative. Feb. 11, 1920, white blood cells 16,400; hemoglobin, 68%. Fed beginning March 9, 1920 (on 64 days, over a period of 115 days) one peptic digest agar slant each of *B. abortus* 80 (certified milk strain) and 14 (fetal membrane strain, 1907) on sliced carrots. Total organisms fed were estimated at 10,000 to 12,000 billion (75-80 billion per slant).

The temperature remained normal during the entire period of observation; there was a drop in the hemoglobin from 68% to 64%. The blood serum gave the following agglutination reactions:

March 9, or the 1st day of feeding—*B. abortus* 80 = 0; *B. melitensis* 11 = 0; 7 = 0.

April 6, or the 28th day of feeding—*B. abortus* 80 = 0; *B. melitensis* 11 = 0.

April 20, or the 42nd day of feeding—*B. abortus* 80 = 1:100 ++; *B. melitensis* 11 = 1:60; 7 = 0.

May 3, or 55th day of feeding—*B. abortus* 80 = 1:100; heated serum also 1:100.

May 17, or 69th day of feeding—*B. abortus* 80 = 1:80.

June 1, or 84th day of feeding—*B. abortus* 80 = 1:100—200.

June 17, or 100th day of feeding—*B. abortus* 80 = 1:80

June 30, or 113th day of feeding—*B. abortus* 80 = 1:100 ++ +; 1:400 ±.

July 10, or 123rd day of feeding—*B. abortus* 80 = 1:1,000 +.

*B. abortus*, hog = 1:800 ++ +; *B. melitensis* 11 = 1:200 ++ +.

*B. melitensis* 20 = 1:200 ++ +; *B. melitensis* 9 = 0;

*B. melitensis* 615 = 0; *B. melitensis* 27 = 0.

July 10, the 123rd day after the first feeding and 8 days after the last feeding, the animal was chloroformed and the tissues cultured.

*Postmortem Examination.*—The cadaver weighed 2,510 gm.; the spleen was of normal size, rather hard and slightly nodular. The follicles were distinctly visible in the fairly dry pulp. The mesenteric, iliac, femoral, prescapular and mediastinal lymph nodes were not visibly enlarged; the liver, kidneys, and other organs appeared normal. The omentum was adherent to the appendix and portions of the colon; one of these adhesions enclosed an abscess, the size of a pea, containing brownish liquid pus. In the colon and appendix wall, numerous nodules the size of split peas, containing *Oesophagostomum brumpti*, were present. Cultures were made from the heart blood, spleen, bone marrow, liver, suprarenals, kidneys, lungs, inguinal, prescapular, mediastinal, mesenteric and iliac lymph nodes and from one abscess in the omentum and one oesophagostomum nodule. The blood plates smeared with the pus of these abscesses and the spleen pulp gave an abundant pure growth of *B. abortus*. All of the agar

slants prepared with crushed liver remained sterile, with the exception of one tube, which showed 2 colonies of *B. abortus*. The other tissues proved to be sterile.

*Exper. 8—Monkey 34.*—*Pithecus syrichta*; female, weight 2,400 gm. Feb. 9 to April 6, 1920, repeated agglutination tests with unheated blood serum were negative for *B. abortus*. On April 6, the hemoglobin was 80% (Sahli) and there were 14,700 white blood cells. The animal was fed on 82 days, over a period of 142 days, one slant each of *B. abortus* 80 and 14, or a total of 14,000 to 16,000 billion bacteria smeared on bread or sliced carrots. Feeding was stopped on July 29, 1920. Weight, blood counts, temperature and agglutination reactions were determined at varying intervals. The temperature never was above 40 C., and the hemoglobin factor showed normal fluctuations. The blood serum always gave negative serum reactions with *B. abortus* and *B. melitensis* antigens. The animal is still alive and in excellent health.

In order to determine the possibility of an elimination of *B. abortus* in the feces of monkeys heavily fed with abortion bacilli, guinea-pigs were inoculated subcutaneously with emulsions of stools, which had been collected and prepared on the 36th, 45th and 52nd days of the experiment. The animals were sacrificed on the 50th to 60th day after the inoculations. Characteristic lesions were absent and no abortion bacilli were recovered from their spleens.

Monkeys 3, 13 and 33 are examples of successful feeding infections with repeated and enormous doses of *B. abortus* administered on carrots, bread or apples. The cultures used in these 3 experiments were of bovine origin and of moderate virulence as judged by the lesions they produced in guinea-pigs. Monkey 3 had been fed previously for a period of 19 days with naturally infected milk, but failed to show signs of infection. The positive cultural findings at necropsy and the specific and high agglutination reactions can be safely attributed to the ingestion of the cultures. Unfortunately, the observations on monkey 3 were somewhat invalidated by the death of the animal from bronchopneumonia. During the summer vacation monkey 13 was attended by an inexperienced caretaker, and lack of proper cleanliness probably resulted in the development of the severe diarrhea. It is, however, evident from the presence of specific serum agglutinins on the 16th day after the beginning of the administration of *abortus* cultures, that the monkey was infected previous to the occurrence of the enteritis. The latter can, therefore, not be considered a contributory factor to the *abortus* infection.

Monkey 34 illustrates the fact that the ingestion of enormous doses of *B. abortus* is not always followed by an infection, provided the appearance of specific agglutinins is to be chosen as the only reliable antemortem criterion. The necropsy findings of its mate, monkey 33,

therefore deserve further discussion. The large intestines and the cecum of this animal were seriously injured by numerous abscesses due to oesophagostomum and the pathologic processes extended into the mesentery and omentum. The majority of these parasitic nodules harbored enormous numbers of viable abortion bacilli. In reviewing the necropsy reports of the monkeys successfully infected by the ingestion of *B. abortus*, it was noted that monkey 13 also showed oesophagostomum nodules, while monkey 3 was not infected. *Special attention has been given to the significance of this observation in subsequent experiments, but true infections with the B. abortus have been recorded in animals which showed no coincidental intestinal lesions due to parasites.* It is therefore only remotely possible that the parasitic lesions predispose to an invasion of *B. abortus* via the alimentary canal in monkeys. In future experiments animals which are free from round worms should be chosen. In our experience with 80 monkeys bought during the last 3 years, such a prerequisite is probably not readily obtainable with the animals at present available on the market. These conditions are mentioned because the British Commission seriously considered the possibility that an absolute continuity of the mucous membrane of the alimentary tract of monkeys may in some cases prevent the development of a feeding infection with *B. melitensis*.

The presumptive evidence of infection has never manifested itself by the presence of clinical symptoms, and the course of the animals' temperatures has been constantly normal. The duration of the incubation period and the course of the infection could therefore only be judged by the appearance and degree of the specific agglutinins, which developed in the blood serum of the monkeys. In this connection it should be emphasized that the serum of monkey 3 on the 20th day and of monkey 33 on the 28th day of feeding failed to agglutinate *B. abortus*, while on the 36th and 42nd day, respectively, the serum of these 2 monkeys was distinctly positive. These observations correspond in many respects to those recorded by Horrocks, Kennedy and Shaw<sup>8</sup> on monkeys infected by the ingestion of *B. melitensis*; approximately 29 days elapsed after the feeding of the cultures before agglutinins were demonstrable in the blood serum. This delayed appearance of the agglutinins lends additional support to the contention that the parasitic processes of the intestines do not favor the tissue invasion of the ingested *B. abortus*. For example, in monkey 3, which had no nodules of oesophagostomum in the colon, agglutinins were absent

<sup>8</sup> See table in Report of Commission, 1907, Part 6, p. 46.

on the 20th day, while similarly in the parasitized monkey 33, 29-30 days elapsed before positive serum tests were obtainable. They were absent at least 28 days after the beginning of the experiment.

In those monkeys in whom serologic evidence of infection was present following the introduction of *B. abortus* by the alimentary route, absolute proof of pathogenicity was obtainable in every instance by the isolation of the organism, at least from the spleen, if not from numerous other viscera.

In order to test the assumption that a strain of *B. abortus*, which is usually fatal in relatively small amounts to guinea-pigs, possesses also greater invasive properties for monkeys than a less virulent strain, two additional experiments were carried out. The *B. abortus* strain employed had been isolated by Dr. J. Traum from the liver of an aborted hog fetus and had proved very lethal for guinea-pigs; it grew profusely on glycerol peptic digest agar and proved biochemically and serologically a typical *B. abortus*.<sup>9</sup>

*Exper. 9—Monkey 43.*—*Pithecus syrichta* (Linn.); female, weight 2,375 gm; healthy, with a blood serum negative to *B. abortus* and *B. melitensis*, was fed on 43 alternate days from June 1, to July 29, 1920 (over 58 days) the growth of one slant of *B. abortus* (hog strain, isolated by Traum) cultivated from the liver of monkey 40. The individual dose averaged 60 billion and for the entire feeding period a total of 2,580 billion bacteria were fed on sliced carrots. The temperature remained normal, but there was a decided loss in weight and specific agglutinins developed in the blood serum as follows:

1st day—weight 2,775 gm.—agglutination with *B. abortus*=0.  
16th day—weight 2,450 gm.—agglutination with *B. abortus*=1:80 + + +.  
37th day—weight 2,425 gm.—agglutination with *B. abortus*=1:400 + + +.  
55th day—weight 2,100 gm.—agglutination with *B. abortus*=1:800 + + + to 1:1,000 + +.  
58th day—feeding stopped.  
65th day—weight 2,225 gm.—agglutination with *B. abortus*=1:600 + + + to 1:2,000 +.

The monkey exhibited no clinical symptoms of infection and was chloroformed on Aug. 4, 1920.

*Postmortem Examination.*—Body well nourished; spleen slightly enlarged and nodular, pulp rather dry; liver normal in size and color; inguinal, mesenteric, subscapular and mediastinal lymph nodes slightly enlarged and slightly pigmented; both kidneys and bone marrow normal. Principle lobe of the left lung contained an echinococcus cyst the size of a plum. In the colon were numerous oesophagostomum nodules with adhesions to the omentum. Cultures were made from the spleen, liver, kidneys, bile, bone marrow, femoral, mesenteric, mediastinal and subscapular lymph nodes, several oesophagostomum abscesses, urine and heart blood. One of 3 plates spread with spleen pulp gave 2 colonies and one plate of 2 smeared with the juice of the mesenteric lymph

<sup>9</sup> Jour. Infect. Dis., 1920, 27, p. 185.

nodes showed 8 colonies of *B. abortus*. All the other cultures were sterile or contained a few staphylococci; on the lung plates *B. bronchisepticus* colonies were present.

*Exper. 10.—Monkey 44.*—*Pithecus syrichta* (Linn.); male, weight 2,800 gm.; healthy with a blood serum negative to *B. abortus* and *B. melitensis*; was fed on 43 alternate days from June 1, 1920, to July 29, 1920 (58 days), the growth of one slant of *B. abortus* (hog strain) isolated from monkey 40. The method of feeding was the same as used for monkey 43. The temperature remained normal; there was a slight loss in weight and the agglutinins developed in the blood serum as follows:

- 1st day—weight 2,800 gm.—agglutination with *B. abortus* = 0.
  - 16th day—weight 2,600 gm.—agglutination with *B. abortus* = 1:40 +.
  - 37th day—weight 2,700 gm.—agglutination with *B. abortus* = 1:100 + + +;
  - 55th day—weight 2,750 gm.—agglutination with *B. abortus* = 1:800 —  
1:1,000 + + +.
  - 58th day—feeding stopped.
  - 65th day—weight 2,925 gm.—agglutination with *B. abortus* = 1:800 + + +.
  - 81st day—weight 3,000 gm.—agglutination with *B. abortus* = 1:800 + + +.  
*B. melitensis* 27=1:600 + + +.
  - 213th day—weight 3,125 gm.—agglutination with *B. abortus* = 1:80 + + +.
  - 325th day—weight 3,100 gm.—agglutination with *B. abortus* = 1:10 + + +.
- On May 5, 1921, the animal was still alive and in excellent condition.

Monkeys 43 and 44 present some interesting features. Judging by the appearance of the serum agglutinins, both monkeys became infected sooner than those which had been fed with less virulent abortion bacilli. The degree of infection did not manifest itself in the necropsy cultures of monkey 43; very few bacteria grew on the plates prepared from the spleen and mesenteric lymph nodes. In connection with the previous discussion particular attention must be concentrated on the fact that the pus material of several oesophagostomum nodules did not contain *B. abortus*; they were either sterile or contained a few staphylococci. Monkey 44 gradually recovered from his infection and regained his original weight. The blood serum agglutinins returned to a low level, but even after a lapse of 325 days, a strongly positive reaction in a dilution of 1:10 was readily obtained. These two experiments indicate that a fairly high percentage of monkeys can be successfully infected via the alimentary tract when large and repeated doses of *B. abortus* are fed on sliced carrots, bread or apples. It has been demonstrated that these infections are associated with strongly positive agglutination reactions and that *B. abortus* can regularly be isolated from the spleen and other organs. The serum of the infected monkeys agglutinates *B. melitensis* cultures in slightly lower dilutions than *B. abortus*. In order to prove definitely the nature of the infection, it is necessary to conduct absorption tests as stated in article 2 of this series.



*Feeding Experiments with Cow's Milk Artificially Infected with B. abortus.*—In a further attempt to investigate the factors involved in the infection through the alimentary canal, 4 monkeys were fed on pasteurized milk, which had been artificially contaminated with varying amounts of 2 bovine *B. abortus* cultures, Nos. 80 and 14. The strains used in these experiments were the same as those used in the successful experiments 6 and 7.

*Exper. 11—Monkey 35.*—*Pithecus syrichta* (Linn.); female, weight, 2,500 gm. Feb. 9 to March 9, 1920, repeated agglutination tests with unheated serums were negative for *B. abortus* and *B. melitensis* 7 and 11; the hemoglobin was 70% and the white blood cells 17,400. The animal received in a pannikin on 52 days over a period of 80 days, 100 cc of pasteurized cow's milk to which had been added on each occasion, just before feeding, the growth of one slant of *B. abortus* 80 and 14. A total of about 10,000 billion bacteria had been given in the milk. As a rule, the monkey received this mixture late in the afternoon, after a starvation period of 18 to 20 hours and usually lapped up from  $\frac{1}{2}$  to  $\frac{3}{4}$  of the amount offered. Inside of from 30 to 60 minutes, the pannikin was ordinarily found to be empty; the animal received then its regular meal.

The monkey never suffered from fever, and gained in weight during the experiment. Agglutination tests made at regular intervals were always negative. The animal is still alive and in good health.

*Monkey 36.*—*Pithecus syrichta* (Linn.); female, weight, 2,450 gm. This animal was treated for the same period in a similar manner as monkey 35, with negative results. The serum tests with *B. abortus* antigens were regularly negative.

*Exper. 12—Monkeys 37 and 38.*—Monkey 37: *Pithecus syrichta* (Linn.); female, weight, 2,075 gm., had been under observation since Feb. 9, 1919; serum tests failed to give positive reactions with *B. abortus* and *B. melitensis* antigens. The monkey was fed by a similar procedure as monkeys 35 and 36 for 44 consecutive days with 100 cc of pasteurized cow's milk, to which had been added the growth of 1/100 slant of *B. abortus* 80 and 14. A total of about 80 to 100 billion bacteria were consumed by the animal during the experiment. She never suffered from fever and never gave specific serum reactions.

On May 14, 1920, or on the 65th day of the experiment, or 21 days after the last feeding of artificially infected milk, the monkey received 0.3 gm. of cascara and the growth of one slant each of *B. abortus* 80 and 14 on carrots for 10 consecutive days; then magnesium sulphate in milk for one day and aloin pills (2, then 4, and then 6 pills of  $\frac{1}{4}$  grain daily) for 7 days, together with carrots artificially infected with *B. abortus* cultures. Neither catharsis nor the appearance of specific agglutinins could be provoked.

From July 8 until Aug. 19 (from the 121st to the 163rd day) the monkey received daily 25 cc of goat's milk collected from goat 2. This milk contained very few abortion bacilli, but possessed a high agglutinin content. During this period of the experiment and for several months subsequently, the monkey never had fever, and the blood serum gave negative agglutinin reactions with *B. abortus* and *B. melitensis* antigens.

*Monkey 38.*—*Pithecus syrichta* (Linn.); a female, weighing 2,000 gm., had been under observation since Feb. 9, 1919; the serum had repeatedly been negative with *B. abortus* and *B. melitensis* antigens. This animal was fed

infected milk in the same manner and on the same dates as monkey 37. Instead of cascara or aloin, tincture rhubarb was administered as a cathartic and 35-40 c.c of goat's milk were given on 42 days over a period extending from May 13 to July 8, 1920.

The monkey never suffered from fever, gained slightly in weight and the blood serum developed no specific agglutinins for *B. abortus*.

It is evident from the data presented that monkeys 35, 36, 37 and 38 failed to become infected. Repeated observations gave the impression that the animals drank a considerable portion of the contaminated food. It was therefore surmised that monkeys 35 and 36 ingested practically the same number of bacteria as those which had been fed with contaminated carrots. The results, however, indicate that this was not the case; in all probability a larger portion of the milk was spilled than was actually recorded, and the total number of *B. abortus* consumed was less than calculated. In no other manner can one explain why organisms ingested in milk should be less pathogenic than those fed on vegetables. As the repeated feeding of milk containing two slants of bacteria was ineffective, it was not anticipated that  $\frac{1}{100}$ th of a slant would be sufficient to cause an infection, so that the negative results in monkeys 37 and 38 were not surprising. There is a remote possibility that these monkeys were refractory. One recalls in this connection that the British Commission succeeded repeatedly in infecting monkeys with *B. melitensis* after the ingestion of a single quantity of mixed milk containing not more than 5,000 specific bacteria. Monkeys 35, 36, 37 and 38 received and probably ingested with impunity many thousand times the amount necessary to cause a typical undulant fever infection.

These experiments demonstrate another fact. The pasteurized milk contained specific agglutinins; its whey clumped *B. abortus* in a dilution of 1:200. The milk of goat 2 (see appendix), which was fed for 42 days to monkeys 37 and 38, was similarly potent. On one occasion, the secretion of the right infected udder agglutinated the *B. abortus* in a dilution 1:2,000. Irrespective of the ingestion of this milk, rich in antibodies, neither of the monkeys presented any specific agglutinins in the blood serum. The mere ingestion of milk containing antibodies does not lead, at least in the monkey, to an appearance of agglutinins in the blood serum; only an invasion of the tissues, limited and transitory as it may be, apparently can be responsible for the formation of specific serum agglutinins.

*Feeding Experiments with Naturally Infected Goat's Milk.*—In this series of experiments an attempt was made to reproduce the conditions

as they were found to exist by the British Commission in their feeding experiments with milk obtained from goats naturally infected with *B. melitensis*. It was originally planned to pass various strains of *B. abortus* successively through the udders of several goats. In this manner it was hoped to transform the bovine into a caprine strain of *B. abortus* and perhaps to produce an organism pathogenically even more closely related to the *B. melitensis* than previous observations had demonstrated. On account of lack of available goats, this phase of the experiment has been temporarily abandoned. The few animals infected with *B. abortus* furnished milk specimens, which were in many respects similar to those found in malta fever goats. The most important observations dealing with the goats are presented in the appendix to this paper. It is emphasized that the milk repeatedly contained as many as 100,000 to 250,000 viable abortion bacilli per c.c. In order to obtain these figures, the milk was plated in suitable dilutions in peptic digest liver agar. It is not unlikely that a large number of viable bacteria failed to grow on the poured plates, irrespective of the fact that the *B. abortus* strains employed grew ordinarily freely in the presence of oxygen. The figures obtained should therefore be considered as averages; it is readily possible that 1-2 million per c.c. would be nearer the true content of the milk than the 250,000 recorded. The mid milk and the strippings, after having discarded the first 5 c.c., were either sterile or gave a pure culture of *B. abortus*. The milk obtained from goat 1, which was infected on 3 occasions with 2 different strains of *B. abortus*, and goat 2, injected once with 3 recently isolated bovine strains were therefore remarkably similar to that ordinarily collected from malta fever goats, and feeding experiments on monkeys, therefore, suggested themselves. The experiments thus far completed are briefly as follows:

*Exper. 13—Monkey 3.*—*Pithecus syrichta*; male, weighing 2,150 gm., July 2, 1919, was well and active; the serum was negative for *B. abortus* and *B. melitensis*. The animal received for a period of 39 days, in a pannikin, 80-200 c.c. of milk obtained from goat 1 (see appendix). A total of 2,090 c.c. of milk were placed in the cage, but the monkey spilled the greater part of the fluid and rarely drank more than 20-30 c.c. at a time. The milk contained from 50-100,000 *B. abortus* per c.c., and the whey of the right udder agglutinated *B. abortus* in a dilution of 1:1,000. The monkey never had any rise in temperature, and the blood serum when tested on July 18 and Aug. 12 failed to agglutinate *B. abortus*.

*Exper. 14—Monkey 19.*—*Macacus cynomolgus*; male, weight 1,650 gm. Sept. 20 to 30, 1919, serum was repeatedly tested for *B. abortus* and *B. melitensis* agglutinins with negative results.

From Sept. 30 to Oct. 30, 1919, the monkey received on 26 week days 60-100 c.c. of goat's milk (goat 1), obtained from the right uninfected half of the

udder. At the beginning of the experiment, the milk from the infected left half of the udder averaged 30-60 c.c., was rich in leukocytes and the sediment of a centrifugalized specimen gave when cultivated on glycerol agar slants numerous colonies of *B. abortus*. Beginning Oct. 12, the secretion of the diseased udder was very scanty, about 5-10 c.c. and toward the end of the experiment (Oct. 14, 1919) the monkey was fed entirely with milk obtained from the right half of the udder, which agglutinated *B. abortus* in a dilution of 1:500. The temperature of the monkey remained normal throughout the experiment. Serum tests conducted on Oct. 10 and 25 were negative for *B. abortus* and *B. melitensis*. Nov. 8, 1919, after showing symptoms of respiratory disease for 3 days, the monkey was found dead.

*Postmortem Examination.*—Body emaciated, tissues dehydrated; lungs, numerous bronchopneumonic areas; spleen tumor; liver and kidneys mottled and viable. Mesenteric and femoral lymph nodes slightly enlarged and soft.

Cultures made on blood plates with the heart blood, spleen, liver, bone marrow, kidneys, urine and bile failed to reveal colonies of *B. abortus*. The lungs, bone marrow, spleen and kidneys revealed hemolytic streptococci.

The experiments clearly indicate that *B. abortus* infection is not readily accomplished in monkeys fed naturally infected goat's milk. Monkeys 3 and 19, which had been fed for 12-39 days, respectively, the milk of goat 1 failed to develop specific serum agglutinins. One, monkey 3, was definitely not refractory because when subsequently fed *B. abortus* on carrots he became infected and showed agglutinins in the serum and bacteria in the tissues. In the light of previous observations, the negative results on these two monkeys were probably due to the following factors: 1. The two *abortus* strains used for the infection of the udder of goat 1 were low in virulence. 2. The numbers of viable abortion bacilli in the milk in comparison to those employed in the carrot feeding experiments were very small. In all probability the daily elimination of bacteria in the milk fluctuated considerably as the figures obtained by direct plating clearly demonstrated. The milk fed to monkey 3 probably contained toward the end of the experiment very few abortion bacilli; thus it was noted that guinea-pigs fed with the same samples of milk failed to contract abortion disease; whereas earlier in the experiment, the milk contained a sufficient number of *B. abortus* to infect guinea-pigs easily by feeding. Moreover, both monkeys rarely consumed more than one-half of the milk offered; monkey 3 receiving at least at the beginning of the experiment milk specimens, which were teeming with abortion bacilli, spilled the greater portion of the fluid. The negative outcome of these two experiments supports the previous conclusions, that monkeys cannot be infected via the alimentary tract with a relatively small

number of abortion bacilli of low virulence even when the contaminated food is administered for more than 30 days. *B. abortus*, therefore, differs fundamentally from *B. melitensis*.

Two additional monkeys, 37 and 38 (see data of experiment 12), were fed for 42 days with milk collected from goat 2. It is known that the secretion of this animal contained very few abortion bacilli: the negative outcome of these feeding experiments is probably due to the factors already discussed.

Being impressed by the high virulence for guinea-pigs of a strain of *B. abortus* recently isolated from a hog fetus, it suggested itself to reinfect goat 1—which was again in a period of lactation having apparently recovered from the original infection with *B. abortus* 80—with this hog strain and to conduct further feeding experiments on monkeys. These tests are briefly as follows:

*Exper. 15—Monkey 40.*—*Pithecus syrichta* (Linn.); female, weight 2,725 gm., had been under observation since Jan. 12, 1920. It had been immunized with

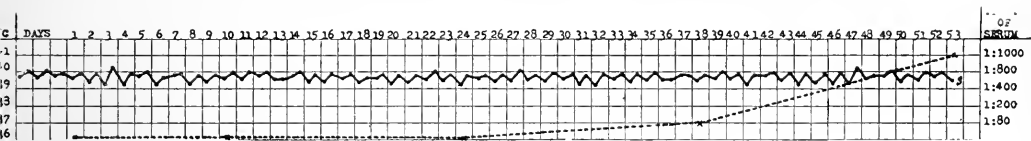


Chart 2 (Monkey 40).—Fed with goat's milk naturally infected with *B. abortus* of porcine origin.

living typhoid bacilli, and the blood serum agglutinated *B. typhosus* in a dilution of 1:8,000, but was negative for *B. abortus* and *B. melitensis*. The hemoglobin was 73% and the white blood cells 12,800.

From March 30 to May 22 the monkey received on 46 successive days 50-100 cc of goat's milk (goat 1), which contained from 20-250,000 abortion bacilli (strain hog) per 1 cc. A total quantity of 2,862 cc of infected milk were offered to the animal in a pannikin. Repeated observations indicated that less than 20 cc were spilled by the animal daily. The method and the time of feeding were those stated for monkey 35. It was estimated that the monkey consumed daily between 1,500 and 2,800 million highly virulent abortion bacilli. On April 23, on the 24th day of feeding, the milk from the right udder of the goat contained 1,500 abortion bacilli per cc and from the left udder 250,000 organisms per cc. May 10, or on the 40th day of the experiment, the milk from the right udder contained 30,000 organisms per cc and from the left udder 180,000 organisms per cc. (For further details see the history of goat 1 in appendix 1.)

The temperature of the monkey showed slight fluctuations during the entire period of observation; it never exceeded the normal average of 40.2 C. (Chart 2). There were no striking clinical symptoms but loss in weight. The figures for the hemoglobin percentages, and the agglutination reactions were as follows:

1st day—weight 2,725—hemoglobin 73%—agglutination with *B. abortus* = 0.  
 10th day—weight 2,700—hemoglobin 73%—agglutination with *B. abortus* = 0.  
 24th day—weight 2,650—hemoglobin 60%—agglutination with *B. abortus* = 0.  
 38th day—weight 2,550—hemoglobin 65%—agglutination with *B. abortus* =  
 1:80 + + +.  
 50th day—weight 2,350—agglutination with *B. abortus* = 1:800 + + +.  
 1:800 + + +.  
 53rd day—weight 2,300—agglutination with *B. abortus* = 1:800—1:1,000 + + +.  
*B. melitensis* 11 =  
 1:400 + + +

May 1, emulsion of stool was injected into 2 guinea-pigs; when sacrificed 3 months later, these animals were free from abortion disease. On the 53rd day, or 2 days after the last feeding of infected milk, the monkey was chloroformed and cultures carefully made.

*Postmortem Examination.*—Cadaver in fair condition; the omentum showed old adhesions to the colon. The spleen weighed 7.2 gm., was distinctly enlarged, and firm and exhibited numerous large follicles. Mesenteric, inguinal, subscapular, mediastinal and iliac lymph nodes were enlarged and hard. Liver and kidneys normal; no intestinal parasites or oesophagostomum nodules. In the lung a few small hemorrhages. Cultures were made from the spleen, inguinal, mediastinal, mesenteric, subscapular and iliac lymph nodes, liver, bile, kidneys, urine, bone marrow, lungs and heart blood. The blood plates smeared with the sections or juices of all the organs were densely crowded with *B. abortus* colonies. The heart blood was enriched in broth and gave on plating a profuse growth of abortion bacilli. The isolated bacteria were promptly agglutinated by a specific abortus serum.

Microscopic examination of the spleen revealed dilated sinuses with a distinct hypertrophy of the sinusoidal cells; the increase of the splenocytes and lymphocytes in the follicles was marked. The malpighian bodies exhibited degenerative changes. In the lymph nodes, desquamated pulp cells and loose endothelial cells in the cortex, together with a pronounced increase of the lymphocytes, attracted attention. Section of the liver, bone marrow and kidneys failed to reveal pathologic changes.

*Exper. 16—Monkey 42.*—*Pithecus syrichta* (Linn.); female, weight 2,425 gm. On June 1, 1920, the animal was in excellent health; the blood serum gave no reaction with *B. abortus* and *B. melitensis*. For 52 days, over a period of 85 days, the monkey was fed daily 100 c.c. or a total of 5,200 c.c. of infected milk of goat 1. At the beginning, the animal refused to drink the milk without the addition of sugar; at least one-half of the contents of the pannikin was spilled. In the second week of the experiment, the monkey consumed practically two thirds of the goat's milk placed in the cage.

June 1, or the 1st day of the feeding, the milk from the left udder of the goat contained 11,800 abortion bacilli per c.c. and from the right udder 2,500 organisms per c.c.; the milk whey of the left udder agglutinated *B. abortus* in a dilution of 1:200 and from the right udder in a dilution of 1:600. On June 25, or on the 25th day of the experiment, the milk of the left udder was sterile, and the milk of the right udder contained 20 abortion bacilli per c.c. July 24, on the 53rd day of the experiment, the milk of the right udder was sterile, while the milk of the left udder contained 10 organisms in the sediment of the centrifugalized 25 c.c. milk sample; the mixed milk of both udders agglutinated *B. abortus* in a dilution of 1:1,000. On August 19, or on the 79th day of the

experiment, the milk of the left udder was sterile; the sediment of 25 c.c of milk of the right udder gave a few colonies; the whey agglutination was positive in a dilution of 1:200.

The serum of the monkey was tested for agglutinins on June 16, July 7 and 28 and August 14, with negative results. There was steady gain in weight and normal temperature throughout the period of observation. The animal died Dec. 20, 1920, while being bled from the saphenous vein.

*Postmortem Examination.*—Status thymo-lymphaticus; old adhesive left pleurisy; no intestinal parasites. Cultures from spleen, mesenteric, inguinal and subscapular lymph nodes, liver, bone marrow, sterile. Serum gave a partial agglutination to *B. abortus* in a dilution of 1:60 in unheated serum; negative in heated serum.

The observations made on monkey 40 present several important facts. The ingestion of virulent abortion bacilli supplied in the naturally infected milk of a goat may lead to a generalized invasion of the tissues and even the heart blood. The intestinal tract of monkey 40 was entirely free from parasites or parasitic lesions. The bacterium penetrated the intact mucous membrane. The duration of the incubation period was more than 30 days. The infection manifested itself in the appearance of specific blood serum agglutinins, a loss in weight and a secondary anemia (Basset-Smith<sup>10</sup> reports the constant occurrence of a secondary anemia in undulant fever) with an increase in lymphocytes, while the animal's temperature remained normal, irrespective of the fact that the tissues were teeming with *B. abortus*. Monkey 42, on the other hand, which also received the naturally infected milk of goat 1, at a later date, showed no signs of an infection throughout the course of the experiment; no traces of agglutinins were demonstrable in its blood serum even up to the date of the termination of the feeding on the 85th day. On the 202nd day, when the monkey died on account of a status thymo-lymphaticus, the unheated serum agglutinated *B. abortus* in a dilution of 1:60, but specific bacteria could not be isolated from the tissues. The failure to contract an infection was probably due to the fact that the monkey refused to take a sufficient quantity of the infected milk when it was particularly heavily contaminated with abortion bacilli. When the monkey had been trained to drink from 20-40 c.c of milk, the total daily number of abortion bacilli consumed was obviously insufficient to cause an infection via the alimentary canal. Exper. 16, therefore, suggests that the ingestion of goat's milk has no predisposing influence in the intestinal invasion of abortion bacilli; it, however, proves by contrast

<sup>10</sup> Jour. Hyg., 1912, 12, p. 497.

with exper. 15 the previously stated conclusion that only enormous doses of abortion bacilli repeatedly ingested are capable of producing infections in monkeys.

*Feeding of Pasteurized Cow's Milk Artificially Reinforced with Autolyzed Abortion Bacilli.*—The observations of Larson, Sedgwick and Ramsey,<sup>11</sup> of Nicholl and Pratt<sup>12</sup> and of Cooledge<sup>13</sup> on the presence of specific (?) agglutinins for *B. abortus* in the serum of man, suggested further feeding experiments on monkeys. Cooledge stated that he caused the appearance of *B. abortus* antibodies in the blood serum of adults by feeding milk, which was naturally infected with *B. abortus* and which contained *B. abortus* antibodies. His explanation that these agglutinins and fixing antibodies resulted from an absorption in the large intestines of those present in an infected cow's milk appeared to be not entirely justified. These conclusions drawn by analogy from the experiments of Ehrlich and Wassermann,<sup>14</sup> dealing with the assimilation of antitoxin through the intestinal canal, should not, as will be shown later, be applied without qualification to this problem. In searching for a better interpretation of these serum reactions in man, it suggested itself to investigate the absorption of bacterial proteins through the intestinal tract of monkeys. Cow's, as well as goat's milk, obtained from infected animals, may contain considerable amounts of autolyzed abortion bacilli, which may even exceed the number of viable invasive organisms. In order to prove that in the monkey at least the development of specific agglutinins is not caused by the assimilation of these substances, the following experiments were carried out:

*Exper. 17—Monkeys 35 and 36.*—These monkeys, used in Exper. 11, with negative results, were fed for 20 days, over a period of 40 days, with pasteurized cow's milk, artificially contaminated with heat killed *B. abortus*, strain "hog." The cultures were prepared on glycerol peptic digest agar, washed off with distilled water and heated for 2 hours at 60 C. Approximately 65 billion dead bacteria were added to the milk of each monkey. Both animals had been trained to drink the milk and spilled less than 20 c.c. of the total amount of 50 c.c. placed in the cage. Serum tests were made every 10 days for 60 days, but no specific agglutinins were recorded.

It is obvious from these experiments that the ingestion of heat killed, partially autolyzed abortion bacilli is not responsible for the appearance of specific agglutinins in the blood serum of monkeys.

<sup>11</sup> Amer. Jour. Dis. Child., 1915, 10, p. 197.

<sup>12</sup> Ibid., p. 2033.

<sup>13</sup> Jour. Med. Res., 1916, 29, p. 459; Mich. Agric. Exper. Station, 1918, No. 33.

<sup>14</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1892, 12, p. 183.



Such immune bodies are apparently only created as a result of a definite tissue invasion by virulent *B. abortus* and their presence is, therefore, indicative of a true infection.

#### EXPERIMENTS WITH *B. MELITENSIS*

In the three preceding papers of this series, it has been pointed out that the distinction between *B. abortus* and *B. melitensis* not infrequently offers great difficulties. Particularly in connection with the *B. melitensis* infection experiments on guinea-pigs, it has frequently been impossible to state without conducting extensive absorption tests whether a strain of gram-negative bacilli which is agglutinated by a potent *B. melitensis* or *B. abortus* serum, is really a *B. melitensis*.

In order to prove the *melitensis* character of two of these strains, which were pathogenic for guinea-pigs on intratesticular injection, the following experiments were carried out:

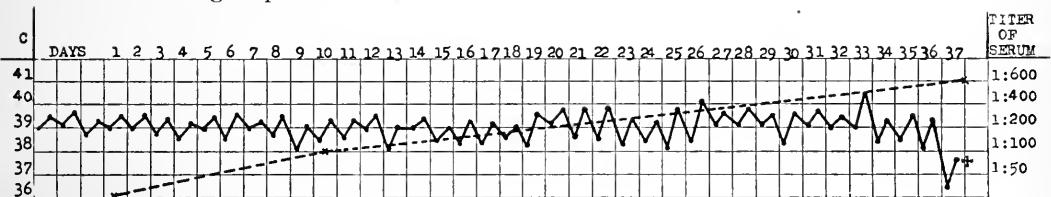


Chart 3 (Monkey 14).—Subcutaneous inoculation of *B. melitensis*.

*Exper. 18—Monkey 14.*—*Macacus cynomolgus*; male, weight 2,830 gm. Aug. 19, 1919, well and active; serum negative for *B. abortus* and *B. melitensis*. Aug. 21, inoculated subcutaneously on abdomen with one-half slant of *B. melitensis* 655 (originally isolated from the precrucial lymph node of guinea-pig 655, which had been infected with a culture obtained from the urine and testes of guinea-pig 614, previously injected with *B. melitensis*, strains 1, 2, 3 and 4). Temperature reaction was distinctly remittant in character (See chart 3). On Sept. 1, or on the 11th day after the inoculation, the blood serum gave the following reactions:

*B. melitensis* 655 = 1:100 + + +

*B. melitensis* 7 = 0

*B. abortus* 80 = 1:40 + + +; 1:100 +

The monkey appeared lethargic and toxic on Sept. 15; he lost in flesh and was dying Sept. 27; killed with chloroform.

*Postmortem Examination.*—Body much emaciated (weight 2,250 gm.), spleen small, hard and fibrous; pulp dark brownish; liver slightly mottled; lymph nodes not appreciably enlarged; femoral lymph nodes rather firm; intestinal content frothy; lungs normal.

Cultures were made from the spleen, heart blood, liver, bile, kidneys, bone marrow, mesenteric, femoral, axillary and mediastinal lymph nodes. *B. melitensis* was recovered from the spleen, right kidney and liver. The blood plates smeared with spleen pulp showed about 100 colonies, those of the right kidney had 20, and those made from the liver gave 2.

The blood serum collected at necropsy, or 37 days after the injection, gave the following reactions:

*B. melitensis* 655 = 1:600 + + +.

*B. abortus* 60 = 1:600 + + +, after absorption with *B. abortus* 80, serum agglutinated *B. melitensis* 1:400 + + +; the reverse test resulted in negative agglutination with both strains.

*B. melitensis* 8, 11 and 2—were not agglutinated in dilution 1:40—1:1,000.

*Exper. 19—Monkey 15.*—*Pithecus syrichta* (Linn.); male, weight 2,010 gm. Aug. 20 to 28, 1919, well and active, temperature normal; serum repeatedly examined for *B. abortus* and *B. melitensis* agglutinins with negative results. Aug. 28, inoculated intravenously with one-quarter slant of *B. melitensis* 655 (precural lymph node of guinea-pig 655) by way of right saphenous vein. Temperature was intermittent in character (see chart 4). On Sept. 1, the monkey was noticeably toxic, and had diarrhea. On Sept. 20 a similar reaction was noticed. The blood serum gave the following reactions:

Sept. 10, 1919, or 13th day after infection:

*B. abortus* 80 = 1:2,000 + + +.

*B. abortus* 14 = 1:600 + + +

*B. melitensis* 655 = 1:400 + + +; 1:2,000 +

*B. melitensis* 7 = 0

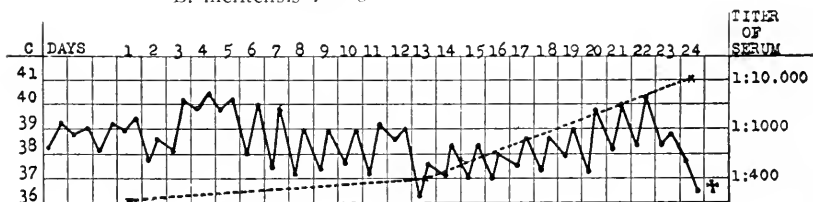


Chart 4 (Monkey 15).—Intravenous inoculation of *B. melitensis*.

Sept. 20, 1919, or 23rd day after infection:

*B. abortus* 80 = 1:20,000 + + +

*B. abortus* 11 = 1:10,000 + + +

*B. melitensis* 655 = 1:10,000 + + +

*B. melitensis* 2 = 1:1,000

*B. melitensis* 7 and 9 = 0

On Sept. 21 the animal was comatose and dying. It was chloroformed.

*Postmortem Examination.*—Body emaciated (weight 1,380 gm.); anus soiled with liquid fecal matter; spleen small, reddish, fibrous pulp; liver light brown and mottled. Mesenteric, femoral and axillary lymph nodes slightly enlarged and soft; bone marrow deep red, like raspberry jam, soft. Blood coagulated rapidly; no intestinal parasites; kidneys light colored. Cultures were made from the spleen, heart blood, liver, kidneys, bone marrow, lung, bile, urine, femoral, axillary and mesenteric lymph nodes. The blood plates smeared with the spleen, bone marrow of left and right femur and one liver lobe were densely crowded with typical colonies; the left lung and urine showed only a few colonies. Absorption tests proved the bacteria to be *B. melitensis*, group 2.

A study of the microscopic preparations revealed a hyperplasia of the sinusoidal cells similar to that usually seen in an early typhoid infection. The spleen showed slight fibrosis and pigmentation, the latter probably the result of an extensive destruction of the blood cells.

1st day—agglutination with	B. abortus	80 = 0
	B. melitensis	27 = 0
24th day—agglutination with	B. melitensis	27 = 1:200 + + +
	B. abortus	80 = 1:400 + + +
94th day—agglutination with	B. melitensis	27 = 1:800 + + +
	B. abortus	80 = 1:800 + + +

Cultures from kidneys, spleen, bone marrow, urine, liver and lymph nodes sterile; plates smeared with pus from the omental abscess were densely covered with *B. melitensis*, group 2, colonies.

In our paper dealing with the serologic classification of the *Brucella* group it was shown that one strain of *B. melitensis*, our No. 20 (obtained from the Lister Institute, Nov. 1919, marked Austria I, received by them from the Royal Army Medical College, Millbank, London), could not be separated by absorption tests from the group I or *B. abortus* group. In order to prove that strain No. 20 is really a *B. melitensis*, the following experiment was carried out:

*Exper. 21—Monkey 45.*—*Pithecus syrichta* (Linn.); male, weight 2,625 gm. Fed July 26 and 29, 1920, the light growth of a 48-hour old slant of *B. melitensis* 20 on carrots, a total of about 70 billion organisms. Under observation for 57 days; slight increase in weight; temperature never above normal. Serum reactions developed as follows:

1st day—agglutination with <i>B. melitensis</i>	20 = 0
10th day—agglutination with <i>B. melitensis</i>	20 = 1:1,000 + + +
15th day—blood cultures sterile	
25th day—agglutination with <i>B. melitensis</i>	20 = 1:2,000 + + +
	<i>B. abortus</i> 80 = 1:600 + + +
57th day—agglutination with <i>B. melitensis</i>	20 = 1:2,000 + + +
	<i>B. abortus</i> 80 = 1:4,000 + + +

Chloroformed on Sept. 25, 1920.

*Postmortem Examination.*—Body in state of excellent nutrition; spleen slightly enlarged and pulp rather soft; other organs normal; no intestinal parasites. Cultures were made from the spleen, liver, kidneys, bile, heart blood, lungs, bone marrow of femurs, mesenteric, inguinal and subscapular lymph nodes. *B. melitensis* (group 1) was recovered from the spleen and mesenteric lymph nodes.

The prompt appearance of agglutinins in the blood of monkey 45 following the ingestion of two slants of *B. melitensis* 20 indicates the high invasion properties of this group of bacteria. Moreover, the presumptive evidence is made absolute by the demonstration of the organisms in the spleen and lymph nodes. Cross agglutination and absorption tests proved the isolated strain to be a true melitensis representative of group 1.

It was not considered necessary to repeat experiment 21; the evidence was conclusive that a true *melitensis* strain differed from a *B. abortus* strain by its marked invasive properties, when administered by mouth. Two feedings were sufficient to parasitize a monkey, but apparently were unable to produce a characteristic febrile reaction. In the *B. abortus* experiments, on the contrary, in spite of the fact that a thousand times as many organisms were ingested at each feeding and these repeated over weeks and months, infection only took place when the dosage was tremendous and the bacteria extraordinarily virulent. This can leave no doubt as to the true nature of strain 20, which, while corresponding serologically to the *B. abortus* group, must be considered a true *melitensis* organism on account of its marked pathogenicity. This observation is quite in accord with those reported by the British Commission,<sup>15</sup> namely, numerous monkeys in their carefully controlled test series manifested neither febrile reactions nor other clinical manifestations, and yet their blood and organs were literally teeming with *B. melitensis*. Future studies on the pathogenicity of the representatives of the *Brucella* group should take these facts into consideration.

One cannot obviously expect the *B. abortus* to be pyrogenic when entering the tissues via the intestinal tract when the highly pathogenic

<sup>15</sup> Report of Commission, 1907, Part 6, p. 67.

and invasive *B. melitensis* quite frequently produces no clinical symptoms. The development of specific agglutinins in the blood serum and the bacterioscopic demonstration of the bacteria in the tissues can be accepted as evidence of true infection.

TABLE 1  
RESULTS OF FEEDING EXPERIMENTS

Number of Experimental Monkey	Duration of "Feeding" in Days	Number of "Feedings"	Dose of <i>B. abortus</i> at Each Feeding	Method of Administration	Evidence of Infection		
					Presumptive		Absolute
					Appearance of Serum Reaction, Days	Reaction of Serum	
1	110	1	1 agar slant	Smeared on sliced carrots or bread	—	Negative	Negative
3	38	36	2 agar slants	Smeared on sliced carrots or bread	36th	1:600-800	Spleen
13	22	11	2 agar slants	Smeared on sliced carrots or bread	16th 22d	1:40-80 +++ 1:80-100	Spleen, mesenteric lymph nodes
33	115	64	2 agar slants	Smeared on sliced carrots or bread	42d 123d	1:100 +++ 1:800 ++	Spleen, liver and parasitic abscesses
34	142	82	2 agar slants	Smeared on sliced carrots or bread	—	Negative	No necropsy
43	58	43	1 agar slant (hog strain)	Smeared on sliced carrots or bread	16th 65th	1:80 1:600-1:2000	Spleen and mesenteric lymph nodes
44	58	43	1 agar slant (hog strain)	Smeared on sliced carrots or bread	16th 81st 213th	1:40 1:800 1:80	No necropsy
35	80	52	2 agar slants	Added to 100 c c of pasteurized milk	—	Negative	No necropsy
36	80	52	2 agar slants	Added to 100 c c of pasteurized milk	—	Negative	No necropsy
37	65	44	2/100 agar slant	Added to 100 c c of pasteurized milk	—	Negative	No necropsy
38	65	44	2/100 agar slant	Added to 100 c c of pasteurized milk	—	Negative	No necropsy
19	38	26	Small number	Naturally infected milk	—	Negative	No necropsy
40	53	46	Large number (hog strain)	Naturally infected milk	38th 53d	1:80 1:1000 +++	All organs, heart blood, bile and urine
42	85	52	Small number (hog strain)	Naturally infected milk	—	Negative	Negative

#### DISCUSSION

The experiments dealing with the intravenous inoculation of *B. abortus* cultures need no special comment; they definitely indicate that *B. abortus* is similar in its action on the monkey to *B. melitensis*. Moreover, the findings serve as a concrete illustration of the prolonged existence of certain bacteria in the tissues of apparently healthy animals.

The infections produced in monkeys by the ingestion of *B. abortus* cultures, however, require further discussion. For the sake of clearness the results of the feeding experiments are shown in table 1. It has been definitely proven that the oral administration of *B. abortus* cultures is followed by an invasion of the tissues and the formation of specific agglutinins, provided enormous doses are fed repeatedly for a period covering at least 11 days. Even when observing these provisions, it becomes evident that some monkeys may be refractory. The feeding of milk either artificially or naturally infected produces invasion only in rare instances. This is probably due to the difficulty entailed in feeding the organisms in sufficiently large dosage by this method. These facts stand in absolute contrast to the experiences of the British Commission when working with *B. melitensis*. In the majority of their experiments, a single oral administration of not more than 5000 *B. melitensis* was sufficient to infect monkeys. It is apparent that this striking difference in pathogenicity cannot be explained on the assumption that the animals in the experiments with *B. abortus* received a smaller number of organisms in the milk than those to whom *B. melitensis* was administered. On the contrary, they ordinarily consumed several thousand times as many abortion bacilli as are necessary to produce a distinct disease when *B. melitensis* is used. The criticism that the elaboration of specific agglutinins, which was used as presumptive evidence of infection, may not occur on account of the continuous feeding and the corresponding absorption of *B. abortus* protein, was carefully considered. This cannot alter the conclusions drawn from these feeding experiments because the serologic studies were not made until the monkeys had been under observation a sufficiently long time after the oral administration of antigens had been discontinued, and, when killed, the animals were cultured thoroughly to demonstrate any exciting focus of infection. These feeding experiments, therefore, offer definite evidence that *B. abortus* is not only serologically different from the predominant groups of *B. melitensis*, but also distinctly less pathogenic.

It appears profitable for comparison to review briefly in this connection the feeding experiments which have been carried out by several workers on guinea-pigs, rabbits and calves. Smith and Fabyean were the first to observe that suspensions of *B. abortus* introduced by mouth produce lesions in guinea-pigs. Schroeder and Cotton<sup>16</sup> noted the development of abortion disease in the same species, when feeding them

<sup>16</sup> Amer. Jour. Dis. Child., 1913, 6, p. 340.

milk of apparently healthy cows that had become chronic carriers of *B. abortus*. Similar observations have been made in our laboratory. The pathologic lesions produced in guinea-pigs fed small quantities of *B. abortus* are slight, but positive cultures can practically always be procured from the spleen and lymph nodes of these animals. A positive agglutination reaction in guinea-pigs that have ingested abortion bacilli has always been indicative of an infection, irrespective of the slight tissue changes noted at necropsy. The same results have not been obtained with the rabbit. Experimental production of an infectious disease via the alimentary canal is peculiarly difficult in this type of animal and only prolonged feeding of large numbers of bacteria may lead to an infection (Besredka, Litch and Meyer). Feeding experiments conducted on guinea-pigs and rabbits are of little significance and practical value when used as presumptive evidence of the pathogenicity of an organism for man.

Some of the generally recognized facts on the susceptibility of calves to *B. abortus* feeding infections deserve consideration. Schroeder<sup>17</sup> and Simms and Miller<sup>18</sup> have determined that calves, at least those which are exposed to infection during the first 3 to 5 months of their lives and are known to have ingested an abundance of infected milk prior to weaning, are immune. The two latter observers base their conclusions on the fact that they are not able to demonstrate the development of specific agglutinins in the serum of these calves subsequent to the feeding of the infected milk. According to Huddelson,<sup>19</sup> serum antibodies specific against *B. abortus* develop occasionally in calves as a result of ingesting naturally infected milk, provided their intestinal tract is made permeable by an existing enteritis. He states, furthermore, that the administration of noninfected milk contaminated with a culture of *B. abortus* for a period of twelve weeks results in the temporary development of complement-fixing antibodies. The demonstration of fixing substances without the coincidental presence of specific agglutinins appears unusual. From a serologic standpoint these findings are remarkable in view of the fact, that old and recent experimental and clinical observations have shown, that the transmission of active or passive immunity through maternal milk can only take place between homologous individuals of certain groups of mammals in the first few days after birth. This occurrence is, however, by no means frequent and certainly does not follow any definite biologic law. This

<sup>17</sup> Report U. S. Live Stock Assn., 1919, p. 125.

<sup>18</sup> Jour. Amer. Vet. Med. Assn., 1921, 58, p. 533.

<sup>19</sup> Michigan Agric. Exper. Station Bull., No. 32, 1916.

peculiar behavior of agglutinins is in striking contrast to the positive nursing experiments of Ehrlich and of Römer with antitoxins. The intestinal tract of young animals can probably absorb agglutinins derived from and present in the milk of the same species more readily than those obtained from heterologous ones. Moreover, the review of Römer<sup>20</sup> on this subject emphasizes the fact that instances of definitely proven transmission of human typhoid agglutinins through the milk of nursing mothers to their infants are not available. These observations are inconsistent with Cooledge's conclusions relative to the absorption of heterologous agglutinins through the intestinal tract of man. If these negative feeding experiments of Schroeder, Simms and Miller on calves are confirmed by other observers, it must be concluded that the intestinal mucosa of these animals is not permeable to homologous maternal milk agglutinins nor for the specific abortion bacilli.

From an epidemiologic standpoint the failure of calves to develop specific agglutinins following the feeding of infected milk is of tremendous interest, if one appreciates that adult bovine animals must be considered exceptionally susceptible to *B. abortus* and that infections are believed to result from the ingestion of this organism. It is obvious that important factors predisposing to a *B. abortus* feeding infection are as yet unknown, but it appears probable that the actual number of ingested bacteria plays an important rôle. The successful feeding experiments of Bang, of MacFadyean and Stockmann and of Zwick and Zeller on adult pregnant cows and goats have been carried out by feeding emulsified placentas or very heavy cultures. In several tests of Zwick and Zeller<sup>21</sup> the oral administration of cultures led rapidly to the development of serum agglutinins; in only one instance, however, to abortion and the demonstration of *B. abortus* in the tissues of the experimental goats and sheep. Similar observations have been made with *B. melitensis* by the British Commission. The feeding of goats with infected milk or cultures was invariably followed by the production of specific agglutinins, but the infection could only be confirmed at necropsy in 40% of the experiments. These observations support the contention that the development of serum agglutinins in animals following the oral administration of *B. abortus* or *B. melitensis* either in culture or in milk is indicative of an infection and that *B. melitensis* is more invasive to ruminants, therefore effective in smaller dosage.

<sup>20</sup> Sommerfeld, Handbuch. d. Milchkunde, 1909, p. 436.

<sup>21</sup> Arb. a. d. k. Gesundheitsamte, 1912, 43, p. 44.



Reference to the dosage necessary for a *B. abortus* infection naturally leads to a discussion of the "virulence" of these bacteria. It must be admitted that our knowledge on this phase of the problem is most incomplete. Guinea-pigs, the usual test animals, exhibit a variable susceptibility, and carefully controlled quantitative experiments have apparently not been carried out. In addition, there is a tremendous degree of variability in the pathogenicity of the different strains. For example, the so-called "hog strain" of *B. abortus* employed in our experiments was highly pathogenic. Guinea-pigs inoculated with 2 to 5 million organisms succumbed regularly to a generalized *B. abortus* infection in from 3 to 4 weeks. The necropsy findings revealed only slight emaciation, a spleen tumor and cloudy swelling of the parenchymatous organs. In the test tube the strains grew readily and abundantly. In comparing a large number of strains and their pathogenicity for guinea-pigs, the impression is gained that growth vigor in the test tubes as a rule runs parallel with a high degree of pathogenicity not only for guinea-pigs, but also for other animals. Opportunity will be afforded to discuss this phase of the problem in a paper dealing with the pathogenicity of *B. abortus* and *B. melitensis* for laboratory animals. In the feeding experiments on monkeys these differences in virulence were decidedly in evidence. In the light of these findings it is justifiable to anticipate that future bacteriologic work may reveal a strain of *B. abortus*, which, in relation to its invasive properties, approaches the *B. melitensis* group more closely than the strains thus far studied.

These possibilities immediately suggest the important question, "Is *B. abortus* pathogenic for man?" In previous papers the literature relative to this problem has been reviewed and certain data presented in which the most striking evidence as to pathogenicity for man rests on the following facts: (a) Mohler and Traum,<sup>22</sup> by inoculating guinea-pigs, found *B. abortus* present in one out of 56 tonsils obtained from children; (b) Larson and Sedgwick,<sup>10</sup> Nicholl and Pratt,<sup>11</sup> and Coolidge<sup>13</sup> demonstrated "specific agglutinins and fixing antibodies in the blood of a small percentage of children and adults, who consumed raw milk; (c) *B. abortus* belongs to the *Brucella* group and is closely related to *B. melitensis*, which is highly pathogenic to man.

The experiments of Mohler and Traum have been repeated by Nicholl and Pratt, with negative results, but aside from this the literature is barren of careful bacteriologic studies of those children's diseases in which *B. abortus* might have been suspected as an etiologic

<sup>22</sup> Twenty-eighth Ann. Report Bur. Animal Ind., Dept. of Agric., 1911, p. 157.

agent. The aforementioned indirect isolation of *B. abortus* from the tonsils must therefore be considered the only instance in which this organism has been isolated from human tissues. The value of the finding is seriously invalidated by the fact that spontaneous *B. abortus* infections occasionally occur in laboratory animals accidentally exposed. Until provision can be made to prevent outside spontaneous infection, a repetition for confirmation of the studies of Mohler and Traum is not feasible.

The demonstration of certain anti-bodies in the blood serum of children and adults deserves further experimental investigation. In view of our negative results in a limited series of about 50 serums obtained from children who consumed raw, certified milk, known to contain *B. abortus*, the positive findings hitherto reported can scarcely be considered constant. The bacteria of the *Brucella* group are quite readily clumped nonspecifically. This subject has been well covered in several papers on *B. melitensis* agglutination by Euzière and Roger, Rouslacroix,<sup>23</sup> Nègre and Raynaud<sup>24</sup> and others. In addition, the occurrence of "inhibition zones" is quite common and the serologist must distinguish between coarse and fine clumping of the bacterial emulsion. Only when these limitations have been carefully noted and the nonspecific agglutinins destroyed by heating to 50 C., as recommended by Nègre and Raynaud, can the reaction be considered specific. The frequent errors in the diagnosis of undulant fever reported in several publications demonstrate that only an agglutination in a dilution above 1:100 is indicative of a true infection. We have not as yet tested a sufficiently large series of human serums by means of the complement-fixation reaction to draw conclusions. The negative findings of Williams and Kolmer<sup>25</sup> on 50 serums demonstrate that even when one's technic is correct suggestive reactions are exceedingly rare. The observations reported in the literature, which have been previously discussed, in addition to the feeding experiments on cattle, guinea-pigs, rabbits and monkeys, definitely demonstrate that heterologous antibodies in cow's milk are probably never absorbed in the large intestines of adult human beings. The solution of the problem of human pathogenicity can probably not be reached by serologic studies alone but must depend on extensive tissue cultures.

Considering the close relationship between *B. abortus* and *B. melitensis*, it seems remarkable that a *B. abortus* disease resembling undu-

<sup>23</sup> Gaz. d. hôp., 1912, 85, p. 289.

<sup>24</sup> Compt. rend. Soc. de biol., 1912, 72, p. 664.

<sup>25</sup> Amer. Jour. Obst., 1917, 75, p. 193.

lant fever has never been recognized in countries in which abortion disease in cattle is common. Several explanations can be offered. *B. abortus* is unquestionably less pathogenic and invasive than *B. melitensis*. It is a well-known fact that great care must be exercised in handling cultures of the *B. melitensis* as bacteriologists working with the organism have become infected in an unusually high proportion of instances as compared with other affections. The laboratory infections reported by Carbone,<sup>26</sup> by Birt and Lamb<sup>27</sup> and by Strong and Musgrave<sup>28</sup> are in striking contrast to the absence of instances dealing with *B. abortus* infections either among laboratory workers, farmers or veterinarians, who unquestionably come in most intimate contact with large numbers of virulent abortion bacilli. Alice C. Evans points out that the number of virulent *B. abortus*, which persist in the milk of cows is not great, probably even so small as to be negligible from the standpoint of food infection. On the other hand, the milk of Malta fever goats is usually teeming with bacteria, in some instances more than 1 million per c c. It is recalled that goats artificially infected with abortion bacilli may discharge enormous numbers of bacteria in their milk, while according to Shaw<sup>29</sup> cows naturally infected with *B. melitensis* (?) may eliminate comparatively few bacteria. The sediment of 10 to 15 c c milk, which had been centrifugalized, gave for one cow on 7 occasions 3-40 colonies, and from another 7-231 colonies. These observations either indicate that the number of *B. melitensis* in cow's milk is relatively small or, what is more likely to be true, that the bacteria demonstrated by Shaw were abortion bacteria, which he could not differentiate with his agglutinating serum from *B. melitensis*. It is probably, however, not so much the amount of virus necessary to cause an infection as the low pathogenicity of the organism for man which explains our freedom from a disease definitely proved to be due to *B. abortus*.

In the paper on *B. abortus* in certified milk it was pointed out that the number of abortus bacilli present in this food was small and the excretion probably variable to a large degree. The assumption that abortion bacilli cannot be demonstrated in cow's milk by direct plating has been refuted by the observations of Huddelson and also of Steck, who isolated the bacteria on ordinary dilution plates. Until our knowledge relative to the actual number of *B. abortus* organisms in

<sup>26</sup> Arch. per le sc. med. Torino, 1904, 28, p. 273.

<sup>27</sup> Lancet, 1899, 2, p. 701.

<sup>28</sup> Philadelphia Med. Jour., 1900, 6, p. 996.

<sup>29</sup> Report of Commission, 1906, Part 4, p. 23.

market or certified milk is based on similar detailed tests as reported for milk of Malta fever goats, it would be unwise to explain our apparent freedom from "abortion disease" by the low number of viable and invasive bacteria. A sudden increase in virulence as shown in one of our goats may lead to a discharge of enormous numbers of abortion bacilli from the udder.

It is impossible to predict and to estimate the consequences of such an event on the human body. An answer to the question—"Is *B. abortus* pathogenic for man?"—must therefore be kept sub-judice even if the evidence thus far collected and critically analyzed encourages the belief that *B. abortus* is under ordinary conditions nonpathogenic.

#### CONCLUSIONS

Following an intravenous inoculation of *B. abortus*, agglutinins develop fairly rapidly in the blood of monkeys. The animals may show an intermittent type of fever and lose weight. At postmortem examination it is possible to recover the organisms from the spleen, lymph nodes and liver, while even on the fourth day after the injection the blood stream is found sterile.

By feeding the growth of glycerol agar cultures of moderately virulent strains of *B. abortus* on carrots, bread or apples in large and repeated doses, it is possible to obtain definite infections in a fairly large percentage of monkeys. The duration of incubation as judged by the appearance of serum agglutinins is always prolonged and at least 30 to 40 days may elapse until such substances can be demonstrated. The infection can be positively proven by the isolation of *B. abortus* from the spleen and occasionally from other tissues.

The repeated oral administration of massive doses of highly virulent *B. abortus* cultures causes a rapid development of specific agglutinins; by the 16th day the serum may be active in a dilution of 1:40-80. Interruption of the feeding leads to a gradual diminution of the antibodies, probably on account of the disappearance of the bacteria from the tissues.

Milk of cows heavily contaminated with cultures of *B. abortus* of low virulence when fed for at least 52 days failed to cause infections. The mere ingestion of cow's milk rich in agglutinins never led to an appearance of these substances in the serum of monkeys.

Milk obtained from goats, which had been experimentally infected with *B. abortus* of low virulence and which contained relatively few bacteria, failed to produce an infection in monkeys even when fed for 12 to 42 days.

By feeding repeatedly the milk of a goat that had been infected by the injection into the udder of a very virulent strain of *B. abortus* of porcine origin, it was possible to infect one monkey. *B. abortus* was isolated at necropsy from all the viscera and the heart blood.

Serum agglutinins specific for the bacteria of the *Brucella* group are formed only in the presence of a definite infection. The ingestion of heat-killed, autolyzed abortion bacilli is antigenically ineffective in monkeys.

Certain strains of *B. melitensis*, which produce characteristic lesions in guinea-pigs resembling those of *B. abortus*, when injected into monkeys intravenously give rise to characteristic temperature, positive agglutination reactions, and the organisms are recoverable at the post-mortem examination from various tissues.

One atypical *melitensis* strain introduced via the alimentary canal was not always pyrogenic, but it stimulated agglutinins and the ingested bacteria persisted for at least 57 days in the spleen and mesenteric lymph nodes.

The microscopic changes produced by a *B. melitensis* or by a severe *B. abortus* infection in the monkey resemble those of an early typhoid infection.

#### SUMMARY

Virulent strains of *B. abortus* in sufficiently large dosage are pathogenic for monkeys.

*B. melitensis* is far more invasive than *B. abortus*. One or two feedings of one-thousandth the amount necessary to cause an infection with *B. abortus* is sufficient in *melitensis* infection to parasitize a monkey.

#### APPENDIX

##### HISTORIES OF GOATS WHICH FURNISHED INFECTED MILK FED TO THE MONKEYS

###### GOAT 1

June 17-20, 1919: About 820 cc of milk were collected daily (3rd month of lactation); Trommsdorff's test, 0.03%; cultures made with the centrifugalized sediment remained sterile; blood serum and whey agglutination tests with *B. abortus* and *B. melitensis* were negative.

June 21: Infected by the injection of the growth of a 24-hour old agar slant of *B. abortus* 80 into the right teat canal. The inoculated half of the udder was not milked until June 28; the daily temperatures of the goat remained normal.

June 28: The right udder was tender, the posterior portion was hard and nodular; 60 cc of milk were collected from the right and 350 cc from the left udder. The milk of the right udder was colostrum like, rich in leukocytes and contained 80-100,000 *B. abortus* per c.c.

June 29—July 7: Daily 100 cc of milk from the right udder; Trommsdorff, 0.05%; the appearance of the milk was normal; slant cultures showed  $\alpha$  B. abortus colonies.

July 8: The blood serum of the goat agglutinated B. abortus 80 in a dilution of 1:1,000; the udder was still nodular and tender; 1 cc of milk from the right udder contained approximately 20,000 abortion bacilli; the milk of the left udder was sterile.

July 17: Collected 100 cc of milk from the right udder; 1 cc of the milk contained 50 viable B. abortus; Trommsdorff, 0.02%.

July 18—Aug. 28: Collected daily 110-150 cc of milk from the right udder.

Aug. 13, 15, 21 and 28: Milk sample cultures for B. abortus remained sterile. Milk collection from Aug. 12—Sept. 13 inclusive fed to 4 guinea-pigs; failed to provoke abortion disease in these animals.

Sept. 1-26: Collected daily an average amount of 126 cc of milk from the right, and an average of 238 cc from the left udder; the whey agglutinated B. abortus in a dilution of 1:80—1:100 + + +.

Sept. 27: Infected the left udder by inoculating the growth of one slant each of B. abortus 80 and 14 through the teat canal.

Sept. 28: The left udder was enlarged, hot and tender; the goat registered a rectal temperature of 40.4 C. and refused to eat.

Sept. 29: The temperature of the goat was normal; the secretion of the left udder, about 5 cc, consisted of curdled milk, very rich in leukocytes; B. abortus was demonstrated by smears and cultures; the right half of the udder secreted a sterile milk.

Sept. 30-October 10: Collected 5-30 cc of yellowish milk from the left and 100-170 cc of milk from the right udder. Agglutination tests of whey were positive in dilutions of 1:100—200 + + +.

Oct. 12: Secretion from the left udder less than 1 cc.

Oct. 20. The goat was bred; secretion from the right udder 50 cc.

Oct. 21-Nov. 10: Milk secretion from the right udder declined from 60 cc to 15 cc and on Nov. 11 there was no longer any secretion from the udder.

March 13, 1920: The goat gave birth to 3 dead but full grown kids; organs of the fetuses were found to be free from B. abortus; milk secretion from both udders was excellent.

March 14 and 15: The goat had retentio placentarum; the blood serum agglutinated B. abortus 80 in a dilution of 1:40; the milk whey of the right and left udders was positive, in a dilution of 1:10; the cultures made from the centrifugalized milk sediment were sterile.

March 25: Infected right udder by inoculating into the teat canal the growth of one slant of B. abortus ("hog's liver"), about 85 billion organisms.

March 26-April 1: The udder was very tender and edematous; the supra-mammary lymph nodes were enlarged and firm. The goat had a toxic appearance, ate little, failed to ruminate and lay in a stupor in the corner of the pen. The coat was rough and the temperature rose daily, in the evening above 40.8 C. The secretion collected from the right udder (not more than 50 cc) was blood-tinged, partially curdled, rich in leukocytes and teeming with B. abortus.

April 2-8: The goat improved clinically; the secretion varied between 35-70 cc from the right and 200-310 cc from the left udder. The temperature of the goat was normal.

April 12: The blood serum of the goat agglutinated B. abortus 80, 1:2000; the milk whey of the left udder was positive in a dilution of 1:800 and the one of the right udder in a dilution of 1:2000 + + +.

April 13-August 28: The goat appeared well, gained in weight and secreted between 60-125 cc milk from the right and 200-300 cc from the left udder. The right half exhibited on palpation numerous nodular indurations; similar but less marked areas were noted in the left udder. Both supramammary lymph nodes were the size of large eggs, very hard and fibrous, but not tender; the leukocyte count of the physically normal milk varied between 6-15,000,000 per cc of secretion obtained from the right and the left udder. The agglutination reactions with serum and milk whey are shown in table 2.

TABLE 2  
AGGLUTINATION REACTION WITH SERUM AND MILK WHEY

Date	Day after Third Infection	Serum Value	Milk Whey Agglutinates	
			Left Udder	Right Udder
March 25, 1920.....	0	1:40	1:10	1:10
April 12, 1920.....	18	1:2000	1:800	1:2000
April 23, 1920.....	29	.....	1:400	1:400
May 10, 1920.....	46	.....	1:1000	1:600
May 22, 1920.....	58	1:1000	.....	.....
June 23, 1920.....	90	.....	1:100	1:600
July 31, 1920.....	128	1:2000	1:1000	1:40

Oct. 25: There was no longer any secretion from the udder. The goat was bred.

March 10: The goat gave birth to 3 mature, healthy kids; the milk collected on March 14, 16, 18 and 20 was sterile; the blood serum agglutinated *B. abortus* in a dilution of 1:20; the milk whey was negative in a dilution of 1:20.

TABLE 3  
NUMBER OF *B. ABORTUS* ISOLATED FROM 1 CC OF THE SECRETION OF EACH UDDER

Date	Day of Third Infection	Number of <i>B. abortus</i> per cc of Milk	
		Left Udder	Right Udder
March 25, 1920.....	0	Sterile	Sterile
March 27, 1920.....	2	Sterile	Dilution too low
April 8, 1920.....	14	50	40,000
April 23, 1920.....	29	1,500	250,000
May 10, 1920.....	46	180,000	30,000
June 1, 1920.....	68	11,800	4,000
June 25, 1920.....	93	0	20
July 24, 1920.....	121	0	10 per loop of sediment
Aug. 19, 1920.....	147	0	3-5 per loop of sediment
Aug. 31, 1920.....	159	0	0

#### GOAT 2

May 27, 1920: Milk whey from both udders gave negative agglutination reactions; blood serum unheated agglutinated *B. abortus* in a dilution 1:40 + + +; heated serum was negative.

June 30: Infected by direct inoculation into the right teat canal of 5 cc representing the growth of one agar slant each of *B. abortus* 60, 100 and 11 (all recently isolated and obtained from Dr. Ward Giltner, East Lansing, Mich.).

July 1-8: The goat was perfectly well; the udder was slightly tender and edematous, but the right udder was not milked.

July 9-18: Daily 15-25 cc of milk were collected from the right and 50-70 cc from the left udder.

July 19: Milk directly plated was sterile; the sediment of 15 cc of milk of each udder gave the following number of colonies; right about 48, left 10 colonies per loopful.

July 24: A similar test gave the following result: right 4, left 12 colonies per loopful of sediment.

July 28: The blood serum agglutinated *B. abortus* 80 in a dilution of 1:2000 + + +; the milk whey of the left udder—1:80 + + +; the milk whey of the right udder—1:2000 + + +.

July 29-Aug. 31: The daily collection of milk gradually dropped from 150 cc to 35 cc. Repeatedly tested, the sediment was found to be sterile, and when inoculated into guinea-pigs failed to provoke abortion disease.

Sept. 10: There was no longer any secretion of milk. Blood serum agglutinated *B. abortus* in a dilution of 1:800 + + +.



# GENERAL INDEX

## A

	PAGE
ACREE, S. F.; MELLON, R. R.; AVERY, PAULINE M., AND SLAGLE, E. A.	
A stable single buffer solution P <sub>H</sub> 1 to P <sub>H</sub> 12	7
ACREE, S. F. See MELLON, RALPH R.	1
Adenoids, diphtheria bacilli and diphtheroids of	62
Adenoids, hemolytic streptococci of	47
Adenoids, influenza bacilli of	55
Adenoids, pneumococci and non-hemolytic streptococci of	51
Alkali reserve of blood in experimental infections	40
Anaerobic bacteria, isolation and cultivation of	227
Anaerobic cultivation	78
Anaerobic fermentation tests, criteria in	321
Anaerobic fermentation tests, tube with seal for	317
Anaphylaxis reactions from milk	200
Antibodies for sheep blood	161
Antimeningococcus serum, opsonic reactivation of	553
Antirabic vaccination with desiccated virus	261
Antitoxins, effects of filtration on potencies of	86
Aqueous humor in normal and immunized rabbits	161
AVERY, PAULINE. See MELLON, RALPH R.	1
See ACREE, S. F.	7
AYERS, S. HENRY; RUPP, PHILIP, AND MUDGE, COURTLAND S. The produc-	
tion of ammonia and carbon dioxide by streptococci	235

## B

B. abortus, pathogenicity of, for monkeys	663
B. botulinus, American strains of	120
B. botulinus associated with fatal pasture disease of horses	114
B. botulinus in canned goods	362
B. chauvœi	615
B. coli, morphologic variation in	591
B. diphtheriae, organisms of B. lactimorbi simulating	513
B. influenzae, growth of	171
B. influenzae, immunologic study of	132
B. influenzae, "satellite" or symbiosis phenomenon of	178
B. lactimorbi in throat cultures simulating B. diphtheriae	513
B. melitensis, pathogenicity of, for monkeys	663
B. pyocyaneus, production of hydrocyanic acid by	73
B. welchii in sugar-free culture medium	344
Bacteria, detection of phenols by	424
Bacterial growth, accessory factors in	171, 178, 187
Bacterial metabolism	227
BARBARIN, R. E. B. chauvœi	615
BECKLER, EDITH; GILLETTE, HELEN, AND PARKER, MARY. Diphtheria car-	
riers among Massachusetts school children	577
BECKWITH, T. D. Studies on the chemotherapy of the experimental typhoid	
carrier condition	495
BELL, WILLIAM H. A method for the detection of phenols produced by	
bacteria	424
BERG, W. N. Effects of filtration on the potencies of antitoxins	86
BERRY, FRED. See PHILLIPS, JAMES MCL.	97

	PAGE
BERWICK, C. COLEMAN. The bacteriology of peridental tissues radio-graphically suggesting infection - - - - -	537
BIGELOW, W. D. The logarithmic nature of thermal death time curves -	528
BITTMAN, FLORENCE R. See NEVIN, MARY - - - - -	429
"Blackhead" in turkeys - - - - -	268
Blood, sugar in, in infections - - - - -	40
Botulinus toxin in contaminated foods - - - - -	287
Botulism, changes in human central nervous system in - - - - -	190
BOYD, A. G. See KAHN, R. L. - - - - -	639
Buffer, ionization constants of glycerophosphoric acid as - - - -	1
Buffer solution $P_H$ 1 to $P_H$ 12 - - - - -	7

## C

CANNON, PAUL R. The effects of diet on the intestinal flora - - -	369
CATHCART, P. H. See ESTY, J. R. - - - - -	29
Chemotherapy of experimental typhoid carrier - - - - -	495
CHESNEY, ALAN M. An immunologic study of <i>Bacillus influenzae</i> -	132
Chilling of body surface, reactions of nasal cavity and post-nasal space to	152
CLARKE, PAUL F., AND MURPHY, ELEANOR J. Virulent micrococcus catarhalis in influenza - - - - -	306
Cocci, gram-negative, in "colds" and influenza - - - - -	462
"Colds," gram-negative cocci in - - - - -	462
Complement-deficient guinea-pigs, serum of - - - - -	611
Complement, fixation - - - - -	630
COOK, M. W., AND STAFFORD, D. D. A study of the gonococcus and gonococcal infections - - - - -	561
COOK, MARJORIE. See KENDALL, A. I. - - - - -	227
Culture mediums containing small percentages of agar - - - - -	390
Culture mediums, ionization constants of glycerophosphoric acid in -	1

## D

D'AUNOY, RIGNEY. Antirabic vaccination with desiccated virus - - -	261
DAVIS, DAVID J., AND MATHEWS, S. A. The bacteriology of the blood of dogs with Eck fistula - - - - -	313
DAVIS, DAVID J. The accessory factors in bacterial growth. IV. The "satellite" or symbiosis phenomenon of Pfeiffer's bacillus ( <i>B. influenzae</i> ) - - - - -	178
DAVIS, DAVID J. The accessory factors in bacterial growth. V. The value of the satellite (or symbiosis) phenomenon for the classification of hemophilic bacteria - - - - -	187
DAVIS, DAVID J. The occurrence of hemolytic streptococci in the normal throat - - - - -	524
Diphtheria bacilli of adenoids and tonsils - - - - -	62
Diphtheria carriers among Massachusetts school children - - - -	577
Diphtheria toxin, effect of, on blood and hemopoietic organs of rabbits	408
Diphtheroids of adenoids and tonsils - - - - -	62
Dysentery-like diseases in children - - - - -	580

## E

ECKER, ENRIQUE E. Comparison of formol and Wassermann reactions in diagnosis of syphilis - - - - -	359
ECKER, ENRIQUE E. Quantitative relations between amboceptor and the serum of complement-deficient guinea-pigs - - - - -	611
Eck fistula, bacteriology of blood of dogs with - - - - -	313

## GENERAL INDEX

701

	PAGE
Empyema, streptococcus, experimental - - - - -	217
ESTY, J. R., AND CATHCART, P. H. The change in the hydrogen-ion concentration of various mediums during heating in soft and pyrex glass tubes - - - - -	29

## F

FABYAN, MARSHALL. See TYZZER, ERNEST EDWARD - - - - -	268
FLEISCHNER, E. C.; VECKI, M.; SHAW, E. B., AND MEYER, K. F. The pathogenicity of <i>B. abortus</i> and <i>B. melitensis</i> for monkeys - - -	663
FOOT, NATHAN CHANDLER. See TYZZER, ERNEST EDWARD - - - - -	268
Formol and Wassermann reaction in diagnosis of syphilis - - - - -	359

## G

GAY, F. P., AND RHODES, BERNICE. Experimental streptococcus pneumonia and empyema. III - - - - -	217
GAY, JEANNETTE N. An experimental study of saline and lipid typhoid vaccines in respect to antigenic and immunizing value - - - - -	417
Genus <i>brucella</i> nov. gen. III - - - - -	663
GILLETTE, HELEN. See BECKLER, EDITH - - - - -	577
Glycerophosphoric acid, ionization constants of - - - - -	1
Gonococcus and gonococcal infections - - - - -	561
Gonococcus types - - - - -	11
GORDON, J. E. The gram-negative cocci in "colds" and influenza. Influenza studies VII - - - - -	462
GOLDMAN, ALFRED; MUDD, STUART, AND GRANT, SAMUEL B. Reactions of the nasal cavity and postnasal space to chilling of the body surface - - -	151
GORDON, J. E. The relationship of the pneumococcus to acute infections of the upper respiratory tract in man. Influenza studies VI - - -	437
Goss, L. W. <i>B. chauvœi</i> - - - - -	615
GRAHAM, ROBERT, AND SCHWARZE, HERMAN R. <i>B. botulinus</i> (Type A) associated with fatal pasture disease of horses - - - - -	114
Gram-negative cocci in "colds" and influenza - - - - -	462
GRANT, SAMUEL B. See GOLDMAN, ALFRED - - - - -	152

## H

HAINES, A. W. <i>B. chauvœi</i> - - - - -	615
HALL, IVAN C. A constricted tube with mechanical seal for anaerobic fermentation tests - - - - -	317
HALL, IVAN C. Criteria in anaerobic fermentation tests - - - - -	321
HALL, IVAN C. See RANDALL, SAMUEL B. - - - - -	344
HAYTHORN, SAMUEL R., AND LACY, GEORGE R. Virulent <i>Treponema pallidum</i> recovered from a stillborn infant after twenty-six hours - - -	386
Heat and complement-fixing antibodies - - - - -	639
HEKTOEN, LUDVIG, AND TUNNICLIFF, RUTH. Opsonic reactivation of anti-meningococcus serum - - - - -	553
Hemophilic bacteria, value of satellite phenomenon for classification of - - -	187
HERMANIES, JOHN. Gonococcus types 2 - - - - -	11
HIRSCH, EDWIN F. Changes in the alkali reserve, sugar concentration, and leukocytes of the blood in experimental infections - - - - -	40
HITCHENS, A. PARKER. Advantages of culture mediums containing small percentages of agar - - - - -	390
Hydrocyanic acid, production of - - - - -	73
Hydrogen-ion concentration of mediums - - - - -	29

## I

	PAGE
Induced morphologic variation in <i>B. coli</i> - - - - -	591
Infections, experimental, alkali reserve, sugar concentration and leukocytes of blood in - - - - -	40
Influenza bacilli in naso-pharynx of tonsillectomized and non-tonsillectomized children - - - - -	59
Influenza bacilli of adenoids and tonsils - - - - -	55
Influenza, gram-negative cocci in - - - - -	462
Influenza, micrococcus catarrhalis in - - - - -	306
Influenza studies - - - - -	437, 462
Intestinal flora, effects of diet on - - - - -	369
ISONO, S. See TSUROMO, M. - - - - -	109
ITANO, ARAO, AND NEILL, JAMES. A microscopic method for anaerobic cultivation - - - - -	78

## J

JACKSON, LEILA. A protozoan parasite in the salivary gland of the dog -	302
JACKSON, LEILA. Negri bodies in the salivary glands and other organs in rabies - - - - -	291
JOHNSON, S. R. See KAHN, R. L. - - - - -	639

## K

KAHN, R. L.; JOHNSON, S. R., AND BOYD, A. G. Complement-fixation. III. The effect of heat on complement-fixing antibodies - - - - -	
KAHN, R. L., AND LYON, D. S. Complement fixation. IV. On the affinity of sheep corpuscles for antishoop hemolysin - - - - -	651
KAHN, R. L., AND OLIN, R. M., JR. Complement fixation. The velocity of fixation of complement in the Wassermann test - - - - -	630
KENDALL, A. I.; COOK, MARJORIE, AND RYAN, MARJORIE. Methods of isolation and cultivation of anaerobic bacteria. Studies in bacterial metabolism. XLIII - - - - -	227
KODAMA, RYUZO. The antibodies for sheep blood and complement of the aqueous humor in normal and immunized rabbits - - - - -	161
KOSER, STEWART A. Trehalose fermentation in the differentiation of the paratyphoid-enteritidis group - - - - -	67
KRUMWIEDE, CHARLES, AND NOBLE, W. CAREY. On the claim that some typhoid-paratyphoid strains survive the milk pasteurization - - -	310

## L

LACY, GEORGE R. See HAYTHORN, SAMUEL R. - - - - -	386
Leukocytes in experimental infections - - - - -	40
LYON, D. L. See KAHN, R. L. - - - - -	651

## M

MATHEWS, S. A. See DAVIS, DAVID J. - - - - -	313
Measles, experimental, in rabbits and monkeys - - - - -	429
Mediums, hydrogen-ion concentration of - - - - -	29
MELLON, RALPH R.; ACREE, S. F.; AVERY, PAULINE M., AND SLAGLE, E. A. The ionization constants of glycerophosphoric acid and their use as buffers, especially in culture mediums - - - - -	1
MELLON, R. R. See ACREE, S. F. - - - - -	7
Metabolism, bacterial - - - - -	227

	PAGE
MEYER, JACOB; PILOT, I., AND PEARLMAN, S. J. Bacteriologic studies of the upper respiratory passages. IV. The incidence of pneumococci, hemolytic streptococci and influenza bacilli (Pfeiffer) in the nasopharynx of tonsillectomized and non-tonsillectomized children - -	59
MEYER, K. F. See FLEISCHNER, E. C. - - - - -	653
Micrococcus catarrhalis in influenza - - - - -	306
Milk, anaphylaxis reactions from - - - - -	200
MITA, K. Dysentery-like diseases (paradysentery, paratyphoid) in children and their causes - - - - -	577
MUDD, STUART. See GOLDMAN, ALFRED - - - - -	152
MUDGE, COURTLAND S. See AYERS, S. HENRY - - - - -	235
MULSOW, F. W. Blanching of the skin by serum injection in scarlet fever - - - - -	557
MURPHY, ELEANOR J. See CLARKE, PAUL F. - - - - -	306

## N

Negri bodies in rabies - - - - -	291
NEILL, JAMES. See ITANO, ARAO - - - - -	78
NEVIN, MARY, AND BITTMAN, FLORENCE R. Experimental measles in rabbits and monkeys - - - - -	429
NOBLE, W. CAREY. See KRUMWIEDE, CHARLES - - - - -	310
NICHOLS, HENRY J. The production of CO <sub>2</sub> by the typhoid bacillus and the mechanism of the Russell double sugar tube - - - - -	82

## O

OLIN, R. M., JR. See KAHN, R. L. - - - - -	630
OLIVER, WADE W. Further observations on a rapid method of pneumococcus typing - - - - -	518
Opsonic reactivation of antimeningococcus serum - - - - -	553
ORR, PAUL F. A rapid method of determining the presence and type of botulinus toxin in contaminated foods - - - - -	287
OSBORNE, THOMAS B. See WELLS, H. GIDEON - - - - -	200

## P

Paratyphoid in children - - - - -	580
PARKER, MARY. See BECKLER, EDITH - - - - -	577
Paradysentery in children - - - - -	580
Paratyphoid—enteritidis group, trehalose fermentation by - - - - -	67
Pasteurization of milk and typhoid-paratyphoid bacilli - - - - -	310
PATTY, F. ARTHUR. The production of hydrocyanic acid by <i>B. pyocyaneus</i> - - - - -	73
PEARLMAN, S. J. See MEYER, JACOB - - - - -	59
PEARLMAN, S. J. See PILOT, I. - - - - -	47, 51, 55
PERKINS, ROGER G., AND SPRENG, D. S. Organisms of <i>B. lactimorbi</i> group found in throat cultures and simulating <i>B. diphtheriae</i> - - - - -	513
Peridental tissues, bacteriology of - - - - -	537
Pfeiffer's bacillus, growth of - - - - -	171
Pfeiffer's bacillus, "satellite" or symbiosis phenomenon of - - - - -	178
Phenols, detection by bacteria - - - - -	424
PHILLIPS, JAMES MCL.; BERRY, FRED, AND SNOOK, J. H. Recovery from rabies, with reports of cases of treatment paralysis and of recovery of animals apparently rabid - - - - -	97
Pigeon, tuberculosis in - - - - -	544
PILOT, I., AND PEARLMAN, S. J. Bacteriologic studies of the upper respiratory passages. I. Hemolytic streptococci of the adenoids - - - - -	47

	PAGE
PILOT, I., AND PEARLMAN, S. J. Bacteriologic studies of the upper respiratory passages. II. The pneumococci and nonhemolytic streptococci of the adenoids and tonsils - - - - -	51
PILOT, I., AND PEARLMAN, S. J. Bacteriologic studies of the upper respiratory passages. III. The influenza bacilli (Pfeiffer) of the adenoids and tonsils - - - - -	55
PILOT, ISADORE. Bacteriologic studies of the upper respiratory passages. V. The diphtheria bacilli and diphtheroids of the adenoids and tonsils - - - - -	62
PILOT, I. See MEYER, JACOB - - - - -	59
Pneumococci in naso-pharynx of tonsillectomized and non-tonsillectomized children - - - - -	59
Pneumococci of adenoids and tonsils - - - - -	51
Pneumococcus and infections of respiratory tract - - - - -	437
Pneumococcus typing - - - - -	518
Pneumonia, streptococcus, experimental - - - - -	217
Protozoan parasite in salivary gland of dog - - - - -	302

## R

Rabies, negri bodies in - - - - -	291
Rabies, recovery from - - - - -	97
Rabies, vaccination, with desiccated virus - - - - -	261
RANDALL, SAMUEL B., AND HALL, IVAN C. The use of <i>B. welchii</i> in the preparation of sugar-free culture medium - - - - -	344
REDDISH, GEORGE F. An investigation into the purity of American strains of <i>B. botulinus</i> - - - - -	120
Respiratory passages, bacteriologic studies of - - - - -	47, 51, 55, 59, 62
Respiratory tract, pneumococcus and infections of - - - - -	437
RHODES, BERNICE. See GAY, F. P. - - - - -	217
RIDDLE, OSCAR. Incidence of tuberculosis in the various organs of the pigeon - - - - -	544
RUPP, PHILIP. See AYERS, S. HENRY - - - - -	235
Russell double sugar tube, mechanism of - - - - -	82
RYAN, MARJORIE. See KENDALL, A. I. - - - - -	227

## S

Salivary gland of dog, protozoan parasite in - - - - -	302
SCALES, F. M. Induced morphologic variation in <i>B. coli</i> - - - - -	591
Scarlet fever, blanching of skin in - - - - -	557
Scarlet fever, serum injection in - - - - -	557
Scarlet fever, spread and persistence of hemolytic streptococci peculiar to - - - - -	91
SCHWARZE, HERMAN R. See GRAHAM, ROBERT - - - - -	114
SEMERAK, C. B. Changes in the human central nervous system in botulism - - - - -	190
SHAW, E. B. See FLEISCHNER, E. C. - - - - -	663
SLAGLE, E. A. See ACREE, S. F. - - - - -	7
SLAGLE, E. A. See MELLON, RALPH R. - - - - -	1
Smallpox, initial exanthem of - - - - -	109
SNOOK, J. H. See PHILLIPS, JAMES MCL. - - - - -	97
SPRENG, D. S. See PERKINS, ROGER G. - - - - -	513
STAFFORD, D. D. See COOK, M. W. - - - - -	561
Streptococcus empyema, experimental - - - - -	217
Streptococci, hemolytic, effects of, on blood and hemopoietic organs of rabbits - - - - -	141
Streptococci, hemolytic, in naso-pharynx of tonsillectomized and non-tonsillectomized children - - - - -	59
Streptococci, hemolytic, in normal throat - - - - -	524
Streptococci, hemolytic, of adenoids - - - - -	47

	PAGE
Streptococci, hemolytic, spread and persistence of - - - - -	91
Streptococci, non-hemolytic, of adenoids and tonsils - - - - -	51
Streptococcus pneumonia, experimental - - - - -	217
Streptococci, production of ammonia and carbon dioxide by - - - - -	235
Sugar concentration of blood in various infections - - - - -	40
Sugar-free culture medium, B. welchii in - - - - -	344

## T

Thermal death time curves - - - - -	528
TONGS, M. S. The effects of hemolytic streptococci on the blood and hemopoietic organs of rabbits - - - - -	141
TONGS, M. S. The effect of the diphtheria toxin on the blood and hemopoietic organs of rabbits - - - - -	408
Tonsils, diphtheria bacilli and diphtheroids of - - - - -	62
Tonsils, influenza bacilli of - - - - -	55
Tonsils, pneumococci and non-hemolytic streptococci of - - - - -	51
Trehalose fermentation - - - - -	67
Treponema pallidum from stillborn infant - - - - -	386
TSURUMO, M., AND ISONO, S. The initial exanthem of smallpox - - - - -	109
Typhoid bacillus, production of CO <sub>2</sub> by - - - - -	82
Typhoid carrier, experimental, chemotherapy of - - - - -	495
Typhoid-paratyphoid strains and milk pasteurization - - - - -	310
Typhoid vaccines, saline and lipoid - - - - -	417
Tuberculosis in pigeon - - - - -	544
TUNNICLIFF, RUTH. Observations on the spread and persistence of the hemolytic streptococci peculiar to scarlet fever - - - - -	91
TUNNICLIFF, RUTH. See HEKTOEN, LUDVIG - - - - -	553
Turkeys, "blackhead" in - - - - -	268
TYZZER, ERNEST EDWARD; FABYAN, MARSHAL, AND FOOT, NATHAN CHANDLER. Further observations on "blackhead" in turkeys - - - - -	268

## V

Vaccines, typhoid, saline and lipoid - - - - -	417
VECKI, M. See FLEISCHNER, E. C. - - - - -	663
Virus, desiccated, antirabic vaccination with - - - - -	261

## W

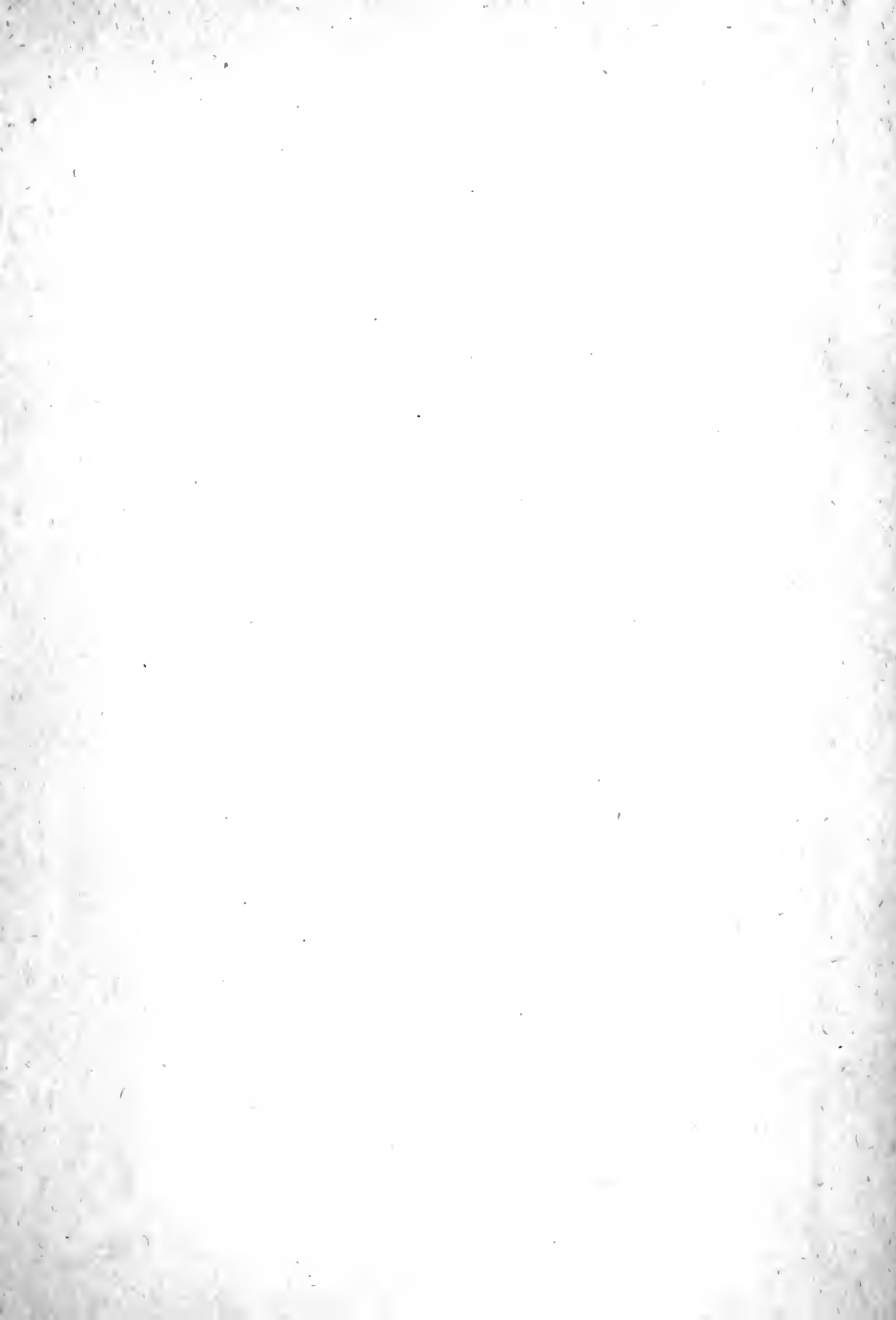
Wassermann reactions and formol in diagnosis of syphilis - - - - -	359
WELLS, H. GIDEON, AND OSBORNE, THOMAS B. Anaphylaxis reactions with purified proteins from milk - - - - -	200
WEISS, HARRY. The thermal death point of the spores of B. botulinus in canned foods - - - - -	362

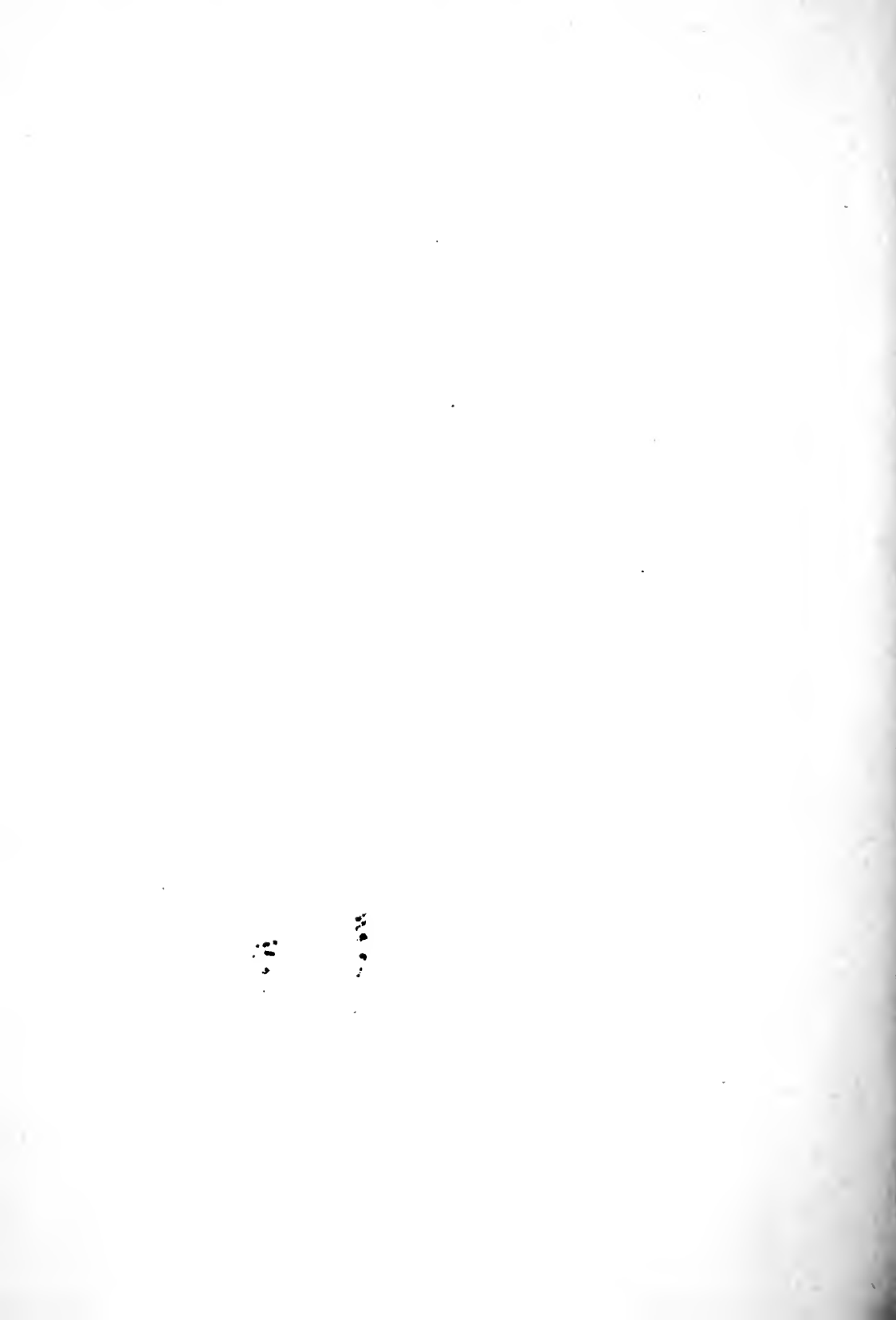












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